OFFICE OF THE SCIENTIFIC DIRECTOR

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LABORATORY OF BEHAVIORAL AND NEUROLOGICAL TOXICOLOGY

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Summary Statement

The scientific efforts of the Laboratory of Behavioral and Neurological Toxicology (LBNT) are directed toward the understanding of the behavioral and neurological effects produced by toxic substances. Special emphasis is placed upon the changes in behavior and neurologic function produced by long-term exposure to low levels of a wide variety of chemical and physical agents present in the environment and to exposure during the development of the nervous system.

The goal of the Laboratory is to obtain a better understanding of neuronal plasticity and nervous system mechanisms responsible for adaptation. This goal reflects our opinion that environmental agents can act to interfere with mechanisms permitting normal plasticity and/or adaptation. General approaches include the following: (1) investigations of the biophysical and molecular biological properties of excitable cells, (2) studies on afferent and feedback mechanisms which influence neuronal plasticity of selected functional systems, (3) studies on the neurobehavioral mechanisms involved in plasticity and adaptive responses to environmental agents, (4) studies on peptides and their effects on biological systems, (5) identification of vulnerable neural circuits by study of neurotransmitter translocations and enzyme analysis, and (6) studies on the functional roles of neurotransmitters or neuromodulators (such as brain peptides) in CNS adaptation following chemical or physical insult.

The research goals of the Laboratory are supported by both intramural scientists and contracted research. The scope of the overall effort is broad involving national and international programs and adjunct appointments. Training opportunities exist for graduate students at nearby universities, foreign and American postdoctoral scientists, and American scientists on sabbatical leave. Current expertise is in the areas of behavior, neuropharmacology, neurochemistry, neuropeptides and neurophysiology.

NEUROBEHAVIORAL TOXICOLOGY

The research of this Workgroup is dedicated to the study of the behavioral and neural effects of environmental agents and, in collaboration with other members of the Laboratory, to the discovery of their mechanism(s) of action. In addition, the group is concerned with the compensatory or adaptive processes activated following exposure to these agents.

Organochlorine chemicals such as chlordecone, p,p'-DDT and lindane are widely used in developing countries and to a lesser extent in some industrialized countries. The purpose of this research is to characterize in an animal model the behavioral and neurological toxicity of chlordecone and study the mechanisms by which chlordecone and other organochlorine chemicals produce their neurotoxic effects. Our experiments have shown in rats that chlordecone-induced tremor can be differentiated from that of p,p'-DDT and permethrin by spectral analysis techniques. Lindane is convulsive, but not tremorigenic, while o,p'-DDT and mirex produce little neurotoxicity in rats. Pharmacological and neurochemical studies have indicated that the origin of the tremor is in the brain stem. While chlordecone and p,p'-DDT are similar in that they increase the turnover of serotonin and norepinephrine, they differ in that p,p'-DDT increases dopamine turnover

and increases levels of excitatory amino acid transmitters; chlordecone has no effects on these measures. Other studies have shown that dilantin markedly attenuates the tremor produced by p,p'-DDT and permethrin and exacerbates the effects of chlordecone and lindane. These data support the interpretation that p,p'-DDT and permethrin act by holding the sodium channel open and indicate that chlordecone and lindane have different mechanisms of action. Future studies will concern the possible role of calcium in the neurotoxicity produced by these agents and the neuropharmacological basis for other neurobehavioral effects produced by these agents.

Organometal (OM) compounds are used for a variety of industrial applications and their trialkyl derivatives produce both neural and behavioral toxicity. The goals of this research are to characterize the time and dose-related neurotoxicity of OMs, examine the potential neurobiological substrates of these effects and study their significance relative to the CNS. The behavior of animals exposed to OMs is similar to that of animals having limbic forebrain lesions. Pharmacological studies implicate catecholaminergic systems in some of the behavioral effects of triethyl lead (TEL). Subsequent studies have examined the effects of OMs on limbic system function. The limbic system modulates behavioral reactivity, emotionality, and memory, and appears to be very sensitive to many toxicants, including OMs. The effects of OMs have been compared to specific cytotoxicants such as AF64-A, 6-OHDA, and DSP-4. Studies to date indicate that the behavioral effects commonly associated with limbic damage appear to be related to a disruption of cholinergic function. These studies suggest that OMs and other neurotoxicants may be useful in the study of the neurobiological basis of some types of metabolic encephalopathies and degenerative disorders.

The development of the central nervous system and its functional output, behavior, reflect a series of maturational events that are highly ordered and precisely timed; the consequences of early disruption can be acute and severe or more subtle and insidious. Neonatal exposure to chlordecone, a prototypic organochlorine, produced tremor and increased reflex reactivity in preweanling rodents, effects similar to those observed in acute toxicity studies with adult animals. Sex-dependent body weight changes persisting into adulthood strongly implicated alteration(s) in the sexual differentiation of hypothalamic centers involved in feeding and weight regulation. Long-term alterations in brain catecholaminergic and serotonergic function were suggested by specific pharmacological challenges. Deficits in retention of learned responses dissociable from nonspecific alterations, such as hyperactivity, were also uncovered. Both short- (hypersecretion) and long-term (depression in basal levels) alterations in circulating and adrenal steroids indicated that chlordecone induced changes in behavioral reactivity and the modulation of memory were produced, at least in part, by acting on the adrenal gland and the feedback regulation of the hypothalamic-pituitary-adrenal axis.

Neonatal exposure to triethyl lead (TEL), a representative organometal, was shown to permanently affect behavioral processes in a manner similar to that observed after damage to the septal-hippocampal system. Long-term behavioral alterations were independent of sensory modality and represented a hyperreactive response of the animal to the test environment. Preferential and permanent destruction of hippocampal pyramidal cell fields (regio inferior) were observed under light microscopy. Pharmacological probes suggested long-term alterations in cholinergic, but not dopaminergic, function.

One common finding in all of the research conducted by this Workgroup is that the nervous system demonstrates considerable resiliency or plasticity following exposure to environmental agents. In one respect, this is important since subtle structural or functional damage may be present in the absence of obvious toxicological endpoints. Examination of behavioral and neural function under appropriate conditions frequently reveals the extent and nature of these alterations. On a more basic level, the compensatory and adaptive processes that are activated by exposure to environmental agents represent a basic feature of neural activity. The study of such processes using neurotoxicants as tools may lead to a better understanding of the functional integration of the nervous system.

NEUROPHARMACOLOGY

The primary goal of the Neuropharmacology Workgroup is concerned with the neurochemical and neuropharmacological bases for the effects of psychoactive compounds and neurotoxicants on behavioral and neurological function. The major research effort has focused on the chemical modulation of neuropeptides and neurotransmitters. The major psychoactive compound studied is haloperidol and the major neurotoxicant studied is chlordecone.

A series of methods employing high performance liquid chromatography have been developed for routine measurement of neurotransmitters and their major metabolites. A variety of radioimmunoassay methods have also been developed for the analysis of levels of peptides and hormones in the brain or tissue fluids. An *in vitro* cell-free translation method and a blot hybridization method have been used for the measurement of biosynthesis of enkephalin in the brain.

The study concerning the possible mechanism underlying chlordecone-induced tremor has suggested that this organochlorine insecticide may increase neuronal excitability by acting on the sodium channel which may be responsible for the increase in the turnover of 5-HT and NE. In turn, the increase in the turnover of these two neurotransmitters may contribute to the expression of chlordecone-induced tremor. Moreover, chlordecone caused a robust increase in the plasma level of ACTH and corticosterone. Preliminary results suggest that the activation of pituitary-adrenal function may be involved in chlordecone-elicited behavioral alterations such as tremor or hyperexcitability.

The studies concerning the modulation of brain enkephalins have revealed that repeated administration of haloperidol or electroconvulsive shock (ECS) increases the mRNA level of this opioid peptide. This suggests that both haloperidol and ECS increase the turnover of enkephalin. This study demonstrates that the gene expression of the enkephalin-system was affected by these treatments. This finding raises an important consideration that gene expression may be the ultimate site of action for various psychiatric treatments.

Alterations of neurotransmitter or neuropeptide function after chlordecone, haloperidol or ECS treatment seen in this study may represent an adaptive process of neuronal systems in response to these insults. Further information from this line of research should provide more insight into the plasticity of different neurotransmitter and neuromodulator systems after exposure to various kinds of environmental insults.

NEUROCHEMISTRY

The major goals of the Neurochemistry Workgroup are: a) determining the biochemical basis for the effects of environmentally prevalent agents on behavior and neurological function, and b) identifying the primary targets of toxic agents and the sequence of events following initial damage by such agents.

An example of the latter of these goals is the study of interactions between toxic organic metals and biologically essential metals.

Many calcium and zinc dependent enzymes are unchanged in triethyl lead treated rats suggesting no across-the-board competition with such divalent metals. This is in accord with in vitro data showing poor competition between triethylead and calcium-binding sites. However, the zinc and copper dependent enzyme superoxide dismutase is elevated in the hippocampus but not in the striatum or frontal cortex of triethyl lead treated rats. In vivo levels of lipid peroxides in treated rats are unchanged as is the level of the selenium-dependant glutathione peroxidase. Thus the elevated superoxide dismutase implies an effective defense against superoxide-induced membrane derangement. A degree of hippocampal membrane damage has been found using trimethyl tin, as judged by altered levels of amino acid uptake. These changes are less pronounced in other brain regions and this selectivity is in agreement with the known morphological susceptibility of the hippocampus to organic tin and lead compounds.

Examples of studies correlating biochemical changes and behavioral derangement are assays of neurotransmitter-related parameters in animals exposed to neurotoxicants.

Changes in levels of neurotransmitter and neuromodulator receptor sites have been examined in organometal-treated rats in an attempt to account for distinctive behavioral changes observed. Triethyl lead is known to have a transient analgesic effect and the time course of this was correlated with a region-specific depression of benzodiazepine binding sites.

The increased seizure susceptibility of triethyl lead exposed animals was found to be reversed by atropine, suggesting the cholinergic system might be involved in this susceptibility. Cholinergic receptors appeared initially depressed and then elevated in the hippocampus of treated rats but more work will be needed to correlate receptor changes and seizure susceptibility on a temporal basis. Depressed receptor levels followed by elevated levels could be explained in terms of an initially hyperactive cholinergic circuit followed by cell death, leading to denervation-related up regulation of post-synaptic receptors. This hyperexcitability followed by neuronal death is thought to be a characteristic effect of excitotoxins on hippocampal tissue. No changes were seen in the receptor levels of the primary excitatory neurotransmitter, glutamate, after triethyl lead treatment.

Other studies on receptor modulation in response to neurotoxic damage include the demonstration of the predictive value of a receptor binding survey using a somewhat understood neurotoxic agent; triorthocresyl phosphate. This agent is known to cause cholinergic hyperactivity by inhibition of acetylcholinesterase in the brain. Hens treated with this agent showed a clear decrease in

levels of muscarinic cholinergic receptors while dopamine, GABA, adrenergic and serotonin receptors were unchanged. Such a selective decrease gives an immediate clue as to the nerve pathways susceptible to damange by triorthocresyl phosphate, and illustrates the value of the receptor approach.

Another study has showed that glucose uptake and amino acid transport mechanisms in the chick retina are readily modulated by altered sensory input. Thus, these techniques may be used to evaluate regional neuronal activity levels in the intact animal.

The biochemical studies of the Neurochemistry Workgroup emphasize the detection of adaptive responses to environmental changes. Such responses can help to explain both the sites of action of toxic chemicals and the resulting behavioral alterations. In this manner the work reflects the laboratory emphasis on neuronal plasticity and cerebral compensatory processes.

PEPTIDE NEUROCHEMISTRY

This work group had focused its efforts on the isolation of two putative neuropeptides. They used highly specific immunological assays for the detection of the peptides coupled with various chromatographic procedures, including high-performance liquid chromatography (HPLC). They were able to purify two new peptides. One, which cross-related with an antibody directed toward the amino-terminal region of physalaemin, was determined to have a mass ion of 866 by mass spectrometry. The amino acid composition of this highly purified material indicated that it was an octapeptide containing two glutamic acids, valine, proline, two aspartic acids, isoleucine, alanine and ammonia. The exact sequence has not yet been established beyond question due to problems inherent in using FAB-mass spectrometry instrumentation. However, based on the specificity of the antibody, a portion of the sequence should be Asp-Pro-Asn. Preliminary studies on the bioactivity of this peptide by Prof. V. Erspamer (Rome) indicates an increase in tonus and phasic motility of guinea-pig illiac smooth muscle with slower onset and relaxation than physalaemin. In rabbit large intestine, it appeared to inhibit tone and motility which was reversed by physalaemin.

The other major area under investigation involves peptides that cross-react with a bombesin-specific antiserum. From the observation that bovine milk contains a bombesin-related peptide, they were able to devise a purification scheme using column chromatography and HPLC. On a small scale, they were able to purify this peptide to apparent homogeneity; however, there was insufficient quantities for chemical analysis. Large scale efforts using powdered milk or fresh skim milk, are under way to obtain microgram quantities of the peptide. In collaboration with Prof. V. Erspamer, this peptide exhibited a bombesin-specific contraction of isolated rat uterus and quinea-pig large intestine and had a particularly evident effect on rhythmic movement. The stimulatory effect was promptly removed by washing. This bovine bombesin was apparently more potent than porcine bombesin-27 and 25-50% as active as amphibian bombesin-14. In a study designed to determine if this peptide existed in mammary tissue, they extracted normal rat mammary tissue and hormone-dependent and-independent carcinogen-induced mammary tumors. Their data indicated that the amount of bombesin immunoreactivity in tumors was 8-12-fold greater than that in normal tissue. HPLC analysis resolved 2 peaks of immunoreactivity. However, they were unable at this time to determine if this peptide is related to that found

in bovine milk. They also analyzed samples of human milk from 9 donors. The results indicated the presence of peptide approximately Mr 5-6000 (that eluted as a single peak on HPLC) as well as immunoreactive high molecular weight material (>20,000) that also cross-reacted with the antibody.

The project goals of this workgroup are to understand the mechanism(s) of action of their new peptides. One physiological approach involves studying the direct effect of the synthetic peptides and antibodies to the peptides on the neuronal plasticity of the developing organism. Furthermore, a collaborative study has been discussed with other workgroups on behavioral modification induced by peptides which would enable an overall view of neuronal changes. Only recently have investigators made inroads in this area of research even though the neuronal localization of many peptides is well known. In fact, the Visiting Fellow that joins their Workgroup in FY 85, having a medical degree, will be able to coordinate these projects and coordinate the biochemical results and approaches with physiologically relevant behavioral studies.

NEUROPHYSIOLOGY

The mission of the Neurophysiology Workgroup is to investigate the changes in neurophysiologic functioning produced by subacute or chronic exposure to low levels of various types of environmental agents. Such research may contribute significantly to environmental health sciences in at least three respects. First, it is expected that research in this area will help discover and refine methods to detect the presence of neurotoxicity following exposure to agents having direct effects on the nervous system. Second, it can aid in determining the portion(s) of the nervous system affected by neurotoxicants. Thus, the neurophysiologic approach to toxicology can play an important role in guiding both neurochemists and neuropathologists with respect to the sites at which they should focus their research. Third, it can give information concerning the biophysical mechanism of action of toxicants by examining their interference with the activity of ion channels.

Some members of this workgroup have long been interested in the electrophysiological basis of cochlear transduction and the elucidation of physiological mechanisms which lead to damage produced by noise and/or chemical agents. Much of their research effort was directed toward studies on ototoxicity of bromate and on the osmolarity of the cochlear fluids under normal and pathologic conditions. Our results indicated that the primary site of action of bromate was on the stria vascularis and in advanced stage of intoxication bromate might affect the hair cells, causing a further suppression of hair cell responses. Systemic administration of glycerin or deionized water did not marked by suppress the cochlear potentials but altered the osmolarity of the cochlear fluids. The osmotic pressure gradient across the cochelar partition was decreased by changing the blood osmolarity. This is a part of a series on proposed experiments in an effort to study adaptive mechanisms and nervous system plasticity.

Other members of the Neurophysiology Workgroup are investigating the neurophysiological effects of organometals. Although the signs and symptoms of organometal toxicity in humans are well known, the precise sites and mechanisms of action are poorly understood. Work in this and other laboratories have provided evidence that the limbic system is a vulnerable site for effects of at least some organometal compounds; particularly trimethyl tin. In addition,

recent studies have demonstrated that triethyl lead, in particular, markedly accelerates the kindling of pentylenetetrazol-induced seizures. The assessment of the neurophysiological bases for pentylenetetrazol-induced kindling and the mechanisms whereby triethyl lead exacerbates it should further our knowledge of excitatory phenomena of the nervous system and how these processes can be altered by neurotoxic substances.

NON-IONIZING RADIATION

The Non-Ionizing Radiation Workgroup has studied the biological effects of 2.45 GHz microwave radiation during the past year. In vitro and whole animal biological systems have been utilized in order to study both the basic interaction mechanisms of microwaves with biological material and the overall effects on more complex specimens i.e., the whole animal. In vitro preparations used have included rat atria, frog sciatic, and lobster giant axons. Frog sciatic nerves have been exposed to continuous wave (CW) and pulse microwave radiation. Rates of fatigue or loss of vitality (the ability of the nerve to continue firing under rapid stimulation) was increased in the nerve exposed to 2.45 GHz at a specific absorption rate of 10 mW/g. In order to determine if sine-wave modulated microwaves had an increasing effect on ionic transport as reported in the literature to occur in chick brain, frog sciatic nerves were exposed to 2.45-GHz microwaves, sine-wave modulated at 8, 16, and 32 Hz. It was found that a 50 mW/g specific absorption rate was required to obtain a loss in vitality with this form of radiation. This result suggests that the nerve vitality is nonlinear with respect to microwave intensity. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field. In order to study this membrane interaction and to obtain a basic understanding of the ionic transport and gating mechanisms in excitable membranes, giant axons of the lobster ganglia are now being studied.

The influence of microwave radiation on cardic tissue using in vitro and in vivo methodologies was also studied. A method for exposing isolated rat atria to microwave radiation has been developed. The data suggest that 2.45 GHz CW microwave radiation of 2 or 10 mW/g has no overt effect on the rate of force of contraction of isolated atria. In addition, the response of atria to drugs was not influenced by microwave exposure. Specifically, the dose response curve for isoproterenol and acetylcholine was not influenced by either 1, 10 or 100 mW/g exposure and the ability of propranolol and atropine to inhibit the isoproteronol and acetylcholine response of rat atria was not altered by these exposure levels. Also, certain biochemical and physiological parameters, which are indicative of cardiac integrity, have been measured in unanesthetized rats during whole body ventral exposure to 2450 MHz CW microwaves. The data suggest microwave exposure of 10 mW/cm for 6 hr has no effect on mean arterial blood pressure or colonic temperature. However, there was a microwave induced bradycardia which was exhibited after 30 min of microwave exposure at 10 mW/cm^2 and persisted throughout the remainder of the 6 hr exposure period. None of the biochemical or hematologic indices examined were influenced by this exposure level. Experiments on the effects of dorsal exposure and on the influence of elevated temperature on the response to microwave radiation have been initiated.

Research has been conducted to determine the effects of exposure to microwave radiation during embryonic development under a contract with N.C. State University. The research uses fertile Japanese quail eggs. These studies are

designed to determine the response of quail exposed during development to stressful conditions at juvenile and adult ages. In all cases the exposures were at a microwave frequency of 2.45 GHz, at an incident power density of 5 mW/cm^2 (specific absorption rate = 4.03 mW/g) and during the first 12 days of development. The hematotogical response of immunized quail was measured in juvenile (5 weeks old) and adult (22 weeks old) quail at 3, 6, and 9 days post-immunization. Only the exposed juvenile female quail elicited a response different from controls. Total reticulocytes were initially depressed and, at 9 days post-immunization, they expressed an increased number of reticulocytes in comparison to controls. The response of quail exposed to microwave radiation in the above manner to hemorrhagic stress also was investigated. The quail were mechanically hemorrhaged to 70% of their calculated blood volume. Circulating erythrocyte and reticulocyte numbers were depressed below controls by 24 hr post-hemorrhage in exposed juvenile and adult females. Lymphocyte numbers were depressed below controls by 24 hr post-hemorrhage in exposed juvenile males. However, lymphocyte numbers were elevated in exposed juvenile males at 72 hr. These data suggested that hematopoiesis is affected by embryonic irradiation and that the effect persists for a long time after hatching (exposure).

The research completed during the past year contributes to the program of LBNT by providing information on the biophysical interaction of an environmental agent with excitable cells. In addition the results help to explain how animals respond, adapt and compensate to a combination of stressors either simultaneoulsy or sequentially imposed. A better understanding of the response of the biological systems and the manner in which the biological systems alter their normal function when exposed to non-ionizing radiation has been obtained.

PERSONNEL

Additions to the Laboratory were: Expert - Dr. P. Chen; Visiting Fellow - Dr. Y. Nakata. Individuals leaving the Laboratory were: Staff Fellow - Dr. S. Swartzwelder; Senior Staff Fellow - Dr. M. Galvin; Accoustical Engineer - Dr. R. Cook; Guest Worker - Dr. H. Ueda; Guest Worker - Dr. G. Gaudino.

OTHER ACTIVITIES

Dr. S. C. Bondy: Adjunct Associate Professor, Department of Pharmacology, University of North Carolina, School of Medicine; Member, NIEHS Radiation Safety Committee; Member, Publications and Education Committee, American Society for Neurochemistry; Member, Editorial Board, Environmental Health Perspectives; Member, Editorial Board, International Journal of Developmental Neuroscience; Member, Editorial Board, Neurotoxicology, Member, Editorial Board, Neurochemical Research; Member, Organizing Committee of the Winter Conference on Brain Research; Contributor to Panel on "Neurotoxicants and Adaptive Responses of the Nervous System," Winter Conference on Brain Research, Steamboat Springs, Colorado, January 1984; Invited paper entitled "Neurotoxicity of Organic and Inorganic Lead Compounds," IBRO Symposium on Neurotoxic Substances and their Impact on Human Health, Lucknow, India, February 1984; Invited paper entitled "Biochemical Evaluation of Neurotoxic Damage," Society of Toxicology Symposium on Mechanisms of Neurotoxicity,

Atlanta, GA, March 1984; Ad Hoc Reviewer, National Science Foundation (Neurobiology Program), Brain Research, Journal of Neurochemistry, Proceedings National Academy of Science, U.S.

Dr. J.-S. Hong: Adjunct Associate Professor, Department of Psychiatry, Duke University Medical School; Invited seminar entitled "Regulation of Brain and Pituitary Enkephalins by Antipsychotic Drugs and Sex Hormones," University of Mississippi; Invited seminar entitled "Regulation of Brain Endorphins by Psychoactive Compounds," National Taiwan University; Invited seminar entitled "Modulations of Brain Enkephalin by Haloperidol," University of Hong Kong; Invited seminar entitled "Biochemical Effects of Organochlorine Insecticides," North Carolina State University; Invited participant, Symposium on Regulatory Peptides, FASEB Meeting; Invited participant, International Symposium on Selective Neurotoxicant; Ad Hoc Reviewer for Brain Research, Science, and Clinical Endocrinology and Metabolism.

Dr. T. Konishi: Ad Hoc Reviewer for National Science Foundation (Sensory Physiology and Perception), Journal of Acoustical Society of America, and Hearing Research; elected Fellow of Acoustical Society of America; received 1984 Award of Merit from Association for Research in Otolaryngology.

Dr. L. H. Lazarus: Adjunct Associate Professor, Department of Pharmacology, School of Medicine, University of North Carolina; Adjunct Member, Cancer Research Center, School of Medicine, University of North Carolina; Ad Hoc Reviewer for Analytical Biochemistry, Cancer Research, Chemico-Biological Interactions, Journal of Biological Chemistry, Proceedings of the National Academy of Sciences, and Science; Invited paper entitled "Physalaemin-like Immunoreactivity in Human Lung Small-Cell Carcinoma," at meeting on Peptide Hormones in Lung Cancer, Marburg, W. Germany, June 1984.

Dr. D. I. McRee: Adjunct Associate Professor, Department of Poultry Science, NCSU; Coordinator US-USSR Cooperative Program on Health Effects of Physical Environmental Factors; Organized workshop and hosted Soviet Delegation to U.S.; NIEHS representative on Interdepartmental Radiation Advisory Committee (IRAC) of the National Telecommunication and Information Administration on Biological Effects of Non-Ionizing Radiation; Representative for DHHS on Interagency Advisory Committee on Electric Field Effects from High Voltage Transmission Lines; American National Standards Institute C95 Committee on Safety Standards for Non-Ionizing Radiation; Appointed to National Research Council Committee on Biological Effects of Non-Ionizing Radiation; Appointed member of IEEE's Committee on Man and Radiation (COMAR); Elected to membership on USA Commission A (Electromagnetic Metrology) of the International Union Radio Science; Appointed to American Technical Program Committee for Joint BEMS-URSI Open Symposium of Electromagnetic Fields and Biological Systems held in Florence, Italy, in August 1984; Appointed to Editoral Review Board of Environmental Health Perspectives; Member, 9-11 Promotion Committee, National Institute of Environmental Health Sciences; Reviewer of manuscripts for Science, The Institute of Electrical and Electronics Engineers, Radiation Research, Health Physics, and Bioelectromagnetics Journal; Reviewer of EPA Criteria Documents and Guidance Documents for Public Exposure to Radiofrequency Radiation; Reviewer of World Health Organization (WHO) Criteria Document; Appointed to five member panel to review Department of Navy's research program in electromagnetic radiation; Project Officer on contract with North Carolina State University.

Dr. C. L. Mitchell: Adjunct Professor, Department of Pharmacology and the Neurobiology Program, University of North Carolina, lectures presented to medical graduate and undergraduate students of the University of North Carolina; Member, Editorial Board, Environmental Health Perspectives; Member, Editorial Board, Neurotoxicology; Member, Editorial Board, Neurobehavioral Toxicology and Teratology; Member, National Institute of Environmental Health Sciences Labor Management Committee; Member, 9 to 11 Promotion Committee, National Institute of Environmental Health Sciences; Chairman, Committee on Methods in Neurobehavioral Toxicology, International Programme on Chemical Safety, World Health Organization; Reviewer of manuscripts for Toxicology and Applied Pharmacology, and Journal of Medicinal Chemistry.

Dr. H. A. Tilson: Adjunct Associate Professor, Department of Zoology, North Carolina State University; Adjunct Associate Professor, Toxicology Curriculum, University of North Carolina; Associate Editor (Field Editor for Neurobehavioral Toxicology) for Neurotoxicology; Member, Editorial Board, Neurobehavioral Toxicology and Teratology; Invited seminar entitled "Mechanisms of Organochlorine-Induced Neurotoxicity," Food and Drug Administration Seminar Series, Divison of Toxicology, Washington, D.C.; Lecturer, Developmental Neurotoxicology, Department of Anatomy, University of North Carolina; Consultant, National Center for Toxicological Research, Jefferson, AR; Consulant, Organization for Economic Cooperation and Development, Ad Hoc Expert Group on Neurotoxicology; Consultant, WHO International Program on Chemical Safety (IPCS); Consultant, Research Project of U.S. Army Medical Bioengineering Research and Development Laboratory; Secretary, Behavioral Teratology Society; Vice-President, Neurotoxicology Speciality Section, Society of Toxicology; Member, Animal Care Committee, National Institute of Environmental Health Sciences; Ad Hoc Reviewer for March of Dimes, National Science Foundation, and Environmental Protection Agency. Reviewer for Brain Research, Experimental Neurology, Journal of Pharmacology and Experimental Therapeutics, Pharmacology, Biochemistry and Behavior, Science, Psychopharmacology, Teratology, Toxicology and Applied Pharmacology, and Toxicology Letters.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25019-03 LBNT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Physalaemin-like Peptide in Mammalian Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lawrence H. Lazarus	Research Chemist	LBNT	NIEHS
Others:	W. E. Wilson	Research Chemist	LBNT	NIEHS
	B. J. Irons	Biological Technician	LBNT	NIEHS
	O. Hernadez	Research Chemist	LEC	NIEHS

COOPERATING UNITS *(if any)*

University of North Carolina, Chapel Hill
University of Rome, Italy

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Peptide Neurochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is continuing under Project Number: Z01 ES 90034-01, Title: Rabbit Stomach Peptide [Physalaemin-like Material (PLIM)] in Mammalian Tissue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 50005-10 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Noise and Ototoxic Agents on Energy Balance and Metabolism in Cochlea

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Teruzo Konishi	Medical Officer (Res.)	LBNT	NIEHS
Others: J. Muratsuka	Visiting Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neurophysiology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.1	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The perilymphatic perfusion with artificial perilymph containing sodium bromate (1mM) was employed in guinea pigs to study the ototoxity of bromate. Changes in the cochlear potentials and electrolyte concentrations in the endolymph were measured in both control and experimental perfusions. The perilymphatic application of bromate resulted in a gradual suppression of the endocochlear potential and sound evoked responses. The recovery of the cochlear potentials was not observed. A substantial decrease in potassium and chloride concentrations and increase in sodium concentration were observed in the endolymph. Our results indicated that the primary site of action of bromate was on the stria vascularis and in advanced stage of intoxication bromate might affect the hair cells, causing a further suppression of the hair cell responses.

PHS 6040 (Rev 1/84) GPO 904-917

PROJECT DESCRIPTION

METHODS EMPLOYED: Clinical reports indicated that bromate poisoning caused irreversible sensorineural hearing loss as well as kidney failure. Preliminary animal experiments attempting to examine the action of sodium bromate indicated that the systemic administration of bromate resulted in suppression of cochlear or microphonics and endocochlear potential. Since the systemic administration of bromate causes severe nephrotoxity and deteriorating general conditions, the precise mechanisms underlying the ototoxicity of bromate are still obscure. As bromate is stable and is not metabolizable in the body, the local application of bromate into the guinea pig cochlea was used to examine the ototoxic action of bromate. Guinea pigs were anesthetized with combination of ketamine and pentobarbital sodium. The endocochlear potential and K^+ or Cl^- activity in the endolymph were continuously measured with double-barreled ion selective electrodes. The cochlear microphonics and compound action potential were also recorded with differential electrodes placed in the basal turn of the cochlea. The perilymphatic space was prefused with artificial perilymph containing 1 mM sodium bromate. The rate of perfusion was 2 µl/min and its duration was 60 min. Samples of the endolymph and perilymph were collected 60 min after the end of perfusion. The concentrations of K^+ and Na^+ were measured with a helium glow photometer and Cl^- concentration was determined using the microtitration technique developed by Ramsay et al. The osmolarity was also measured employing the freezing point depression.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Cochlear potentials.

 In control experiments in which the perilymphatic space was perfused with artificial perilymph the endocochlear potential showed a tendency to increase during perfusion and its mean increase was 5.9% at the end of perfusion. During the 60 min period after perfusion the endocochlear potential gradually returned to the preperfusion level. At the end of perfusion the normalized cochlear microphonics and compound action potential was 86.1% and 110.9% respectively.

 When the perilymphatic space was perfused with artificial perilymph containing 1 mM of sodium bromate, the endocochlear potential showed an initial increase followed by a gradual decrease. The mean loss of the endocochlear potential was 12.6% at the end of perfusion. The endocochlear potential continued to decrease and no recovery was observed after the perfusion. The mean loss was 50.6% 60 min after the end of perfusion. The cochlear microphonics and compound action potential decreased gradually during perfusion and their losses were 12.6% and 50.7% respectively at the end of perfusion.

2. Electrolyte concentrations and osmolarity in the endolymph.

 The K^+ and Cl^- activities in the endolymph measured with ion selective electrodes were 112.8 ± 2.06 mEq/l and 99.2 ± 0.9 mEq/l respectively. In control perfusion no substantial changes in K^+ or Cl^- activities in the endolymph were observed during and after perilymphatic perfusion.

During the perilymphatic perfusion with bromate solution the K^+ activity in the endolymph increased slightly and the mean increase was 6.1 mEq/l at the end of perfusion. Thereafter the K^+ activity in the endolymph decreased continuously and its loss was 10.5 ± 4.2 mEq/l 60 min after the end of perfusion. The increase of Cl^- activity in the endolymph was 4.6 ± mEq/l at the end of perfusion. The loss of the Cl^- activity measured 60 min after the end of perfusion was 7.4 ± 1.9 mEq/l.

The K^+, Na^+ and Cl^- concentrations in the endolymph sampled 60 min after perfusion with the control solution were 157.8 ± 4.3 mM, 0.4 ± 0.1 mM and 142.2 ± 1.3 mM respectively which showed no significant changes as compared to the endolymph electrolyte concentrations in nonperfused cochlea. The electrolyte concentrations in the endolymph showed marked decreases in perfusion with bromate. The endolymph K^+ concentration was 144.0 ± 4.5 mM, Na^+ concentration 2.6 ± 0.5 mM and Cl^- concentration was 126.2 ± 1.2 mM. The osmolarity of the endolymph decreased to 282.7 ± 3.8 mOsm/kg.

These findings suggest that the primary site of ototoxic action of bromate is the stria vascularis and advanced bromate intoxication also affects the hair cells. We plan to study the dose-response relationship of the cochlear potentials and electrolyte concentrations in the cochlear fluids. In addition we plan to examine effects of bromate introduced into the endolymph on the cochlear potentials and electrolyte composition of the endolymph.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The specific ototoxicity of bromate will be revealed by the results obtained by these studies together with the morphological studies which are in progress. The present study will make a significant contribution for understanding the ototoxicity of bromate. This project will be a significant part of the overall Institute Program which provides an essential knowledge base on the impact of environmental factors on human health.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 50015-10 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Microwaves on Neural Response

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Donald I. McRee	Research Physicist	LBNT	NIEHS
Others: Clifford L. Mitchell	Supv. Pharmacologist	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Non-Ionizing Radiation

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, NC

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	0.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Frog sciatic nerves have been exposed to continuous wave (CW) and pulse microwave radiation. Rate of fatigue or loss of vitality (the ability of the nerve to continue firing under rapid stimulation) was increased in the nerve exposed to 2.45 GHz at a specific absorption rate of 10 mW/g. In order to determine if sine-wave modulated microwaves had an increasing effect on ionic transport as reported in the literature to occur in chick brain, frog sciatic nerves were exposed to 2.45-GHz microwaves sine-wave modulated at 8, 16, and 32 Hz. It was found that a 50 mW/g specific absorption rate was required to obtain a loss in vitality with this form of radiation. This result suggests that the nerve vitality is nonlinear with respect to microwave intensity. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field. In order to study this membrane interaction and to obtain a basic understanding of the ionic transport and gating mechanisms in excitable membranes, the giant axons of the lobster ganglia are now being studied.

PROJECT DESCRIPTION

METHODS EMPLOYED: Isolated neurons such as the sciatic nerve of the frog, lobster ganglia, and aplysia abdominal ganglia will be exposed to CW, pulsed and modulated microwave radiation at levels which produce different electric field intensities inside the tissue. The interaction of these electric fields with nerve function including membrane gating and ionic transport will be investigated. These results could provide a better basic understanding of the function of excitable membranes.

MAJOR FINDINGS AND PROPOSED COURSE: We have determined that microwave radiation does affect isolated sciatic nerves of the frog. This effect was seen as a loss in vitality when the nerves were rapidly stimulated. Use of various modes of delivery of microwaves (continuous wave, pulse wave, and frequency modulated waves) indicate that the effect is nonlinear involving rectification across the membrane. This type of nonlinear behavior would be expected if the neural membrane is acting as a diode-like detector of the microwave field.

In order to investigate the mechanism for the loss in vitality of frog sciatic nerves exposed to CW microwaves, nerves were treated with ouabain before being exposed. In this case no difference in loss of vitality was observed between the exposed and nonexposed nerves. Since quabain blocks the activity of the Na^+-K^+ pump, this result suggests that the microwave effect on nerve vitality are associated with the decay of ionic gradients normally maintained by active transport.

Further studies will be aimed at determination of the interaction mechanisms producing the effect and to gain basic information on membrane function (gating, transport, permeability). The lobster giant axon will be used in these studies. If the lobster nerve is affected in the same way as the frog sciatic nerve, experiments will be carried out to explain why the change is occurring. It is believed that the loss in vitality of the nerve is due to an interaction with the long-term regulatory process - such as the maintenance of adequate ionic concentration gradient across the membrane - rather than an interference with the action potential firing mechanism. Therefore, our initial approach will be directed at effects on ionic transport through the membrane and ionic depletion. Membrane channel blockers and radioactively labelled ions (uptake and perfusion technique) will be used to evaluate these possible mechanisms.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND PROGRAM OF THE INSTITUTE: This research will contribute to the base of knowledge on the physiology of excitable membranes and will provide information on how membrane function is altered when perturbed by an environmental agent - in this case, microwave radiation. This research is directed toward the mission of the Institute to conduct biomedical research concerned with the effects of physical environmental agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50038-06 LBNT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of 2450 MHz Microwave Radiation on the Cardiovascular System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Michael J. Galvin	Senior Staff Fellow	LBNT	NIEHS
Others: Donald I. McRee	Research Physicist	LBNT	NIEHS

COOPERATING UNITS *(if any)*

Duke University

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Non-Ionizing Radiation

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of this project are to determine the influence of microwave radiation on cardiac tissue using in vitro and in vivo methologies. A method for exposing isolated rat atria to microwave radiation has been developed. The data suggest that 2.45 GHz CW microwave radiation of 2 or 10 mW/g has no overt effect on the rate of force of contraction of isolated atria. In addition, the response of atria to drugs was not influenced by microwave exposure. Specifically the dose response curve for isoproterenol and acetylcholine was not influenced by either 1, 10 or 100 mW/g exposure and the ability of propranolol and atropine to inhibit the isoproteronol and acetylcholine response of rat atria was not altered by these exposure levels. The influence of microwave radiation on the response of isolated atria to other cardiotonic drugs are being undertaken. Also, certain biochemical and physiological parameters, which are indicative of cardiac integrity, have been measured in unanesthetized rats during whole body ventral exposure to 2450 MHz CW microwaves. The data suggest microwave exposure of 10 mW/cm^2 for 6 hr has no effect on mean arterial blood pressure of colonic temperature. However, there was a microwave induced bradycardia which was exhibited after 30 min of microwave exposure at 10 mW/cm^2 and persisted throughout the remainer of the 6 hr exposure period. None of the biochemical or hematologic indices examined were influenced by this exposure level. Experiments on the effects of dorsal exposure and on the influence of elevated temperature on the response to microwave radiation have been initiated. No data are available for these experiments.

PROJECT DESCRIPTION

METHODS EMPLOYED:

a. Isolated rat atria maintained at either 22° or 37°C were exposed to microwave radiation at specific absorption rates of 1, 2, 10 and 100 mW/g and rate and contractile force were monitored. For each experiment 2 pairs of atria were used, one control and one exposed, which were placed in specially designed tubes located in a waveguide exposure apparatus. In addition, the response of the tissue to sympathetic and parasympathetic agents has been determined durir microwave exposure. The drugs used included propranolol, isoproterenol, acetylcholine and atropine.

b. Adult male rats were exposed to whole body microwave radiation of 2 and 10 mW/cm^2 at carefully controlled temperatures and exposure levels. Using a specially designed irradiation chamber, rats were exposed either dorsally or ventrally, and certain hemodynamic (blood pressure, heart rate), hematologic and biochemical parameters were measured during 6 hr microwave exposure. The temperature, humidity and noise level in the exposure chamber were maintained at either 23° or 27°C, 60 and 70 dB respectively during the experimental period.

MAJOR FINDINGS AND PROPOSED COURSE:

a. The data indicate that the exposure rates used (2 and 10 mW/g) have no overt effect on the rate or force of contraction of isolated atria at either incubation temperature. At 22°C the rate of contraction was 12 beats per minute for both the control and exposed atria. Atria maintained at 37°C had a rate of contraction of 215 beats per minute, and was also unchanged by microwave exposure. These experiments have been extended to examine the response of atria to drugs during microwave exposure. Dose response curves to various sympathetic and parasympathetic acting agents were determined for atria exposed to 1, 10, or 100 mW/g. Microwave exposure had no influence on the atrial response to the tested agents. In addition, the action of sympathetic and parasympathetic antagonists on exposed atria were not altered by microwave exposure.

b. The data from these experiments indicate that ventral microwave radiation (10 mW/cm^2) at 23°C, induced a bradycardic after 1 hr of exposure. Specifically, in the sham exposed rats the mean aterial blood pressure (MABP), heart rate (HR) and colonic temperature were 120 ± 10 mmHg, 300 ± 30 BPM, and 37.5 ± 0.8° respectively. Six hour exposure to 10 mW/cm^2 microwave radiation had no influence on MABP or temperature. However, there was a highly significant reductio in heart rate which was evident within 60 min after the beginning of the exposure and persisted for the remainder of the experiment. In addition, a number of hematologic and biochemical parameters were examined during the exposure period. No differences between the sham (0 mW/cm^2) or exposed (10 mW/cm^2) groups were noted for any of these parameters.

Experiments are now being conducted which examine the influence of whole body dorsal microwave exposure as well as experiments on the influence of elevated environmental temperature of 27°C on these parameters. In addition, the in vivo responses to autonomic nervous system agonists will also be examined during ventral or dorsal microwave exposure.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The potential health effects of microwave radiation in the environment is of interest to NIEHS. This project is designed to provide a comprehensive and integrated study of the possible effects of microwave radiation on the cardiovascular system. By using in vivo techniques microwave interactions with cardiovasular system can be evaluated more effectively. This research on the effects of microwaves on the cardiovascular system is directed toward the mission of NIEHS to determine the health effects of physical factors in the environment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 50076-03 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Noise and Drugs on Water Control of the Cochlear Fluids

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Teruzo Konishi	Medical Officer (Res.)	LBNT	NIEHS
Others:	Y. Muratsuka	Visiting Fellow	LBNT	NIEHS
	H. Ueda	Guest Worker	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neurophysiology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.7	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to elucidate the mechanisms of water movement across the cochlear partition. Systemic injection of glycerin or deionized water was used to induce alterations in the osmolarity of the blood serum in guinea pigs. The cochlear potential including the endocochlear potential did not show marked suppression when the osmolarity of the cochlear fluids was elevated or lowered. Under normal conditions the osmolarity is significantly higher in the endolymph than perilymph. When the blood osmolarity was altered, the osmotic gradient across the cochlear partition decreased.

These studies are used as base line data to which water control in the cochlear fluids under pathologic conditions can be compared.

PROJECT DESCRIPTION

METHODS EMPLOYED: The cochlear fluids are in the state of dynamic balance with continuous exchange of ions and metabolites between the cochlear fluids and their environment. It has been speculated that ototoxic agents may lead to local or systemic disturbance in the ion and water exchange mechanisms. No quantitative changes in the osmotic pressure of the cochlear fluids or hydrostatic pressure gradient across the cochlear partition following administration of various ototoxic drugs or chemicals have been reported. The purpose of this study was to investigate the osmotic relation between the cochlear fluids, blood and cerebrospinal fluid (CSF).

Guinea pigs were anesthetized with combination of ketamine and pentobarbital sodium. The cochlear microphonics (CM) and compound action potential (AP) were recorded with differential electrodes placed in the basal turn of the cochlea. The double-barreled ion selective microelectrodes were used to record the endocochlear potential (EP) and ionic activity in the endolymph continuously. Similar ion selective electrodes were utilized to record ion activity in the perilymph. Samples of the cochlear endolymph and perilymph were also collected. The CSF was collected by puncture of the atlantooccipital membrane. Osmolarity of the cochlear fluids and CSF was determined using the freezing point depression. Osmolarity of blood serum was determined with a vapor osmometer. The K^+ and Na^+ concentration was measured with a helium glow photometer and the Cl^- concentration was determined by the microtitration method.

Osmolarity of the blood was altered by intravenous injection of 3 g/kg glycerin or intraperitoneal injection of 1 ml/10 g BW of deionized water.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Nontreated guinea pigs.

 The osmolarities of the perilymph in the scala vestibuli and the scala tympani were 290.1 ± 5.2 mOsm/kg and 290.3 ± 4.4 mOsm/kg respectively. The osmolarity of the endolymph was 301.5 ± 5.3 mOsm/kg which was significantly higher than perilymph. The osmolarity of CSF was 289.1 ± 3.0 mOsm/kg and the blood osmolarity was 290.2 + 5.9 mOsm/kg.

2. Guinea pigs with hyperosmolarity induced by glycerin injection.

 a) Cochlear potentials.

 The EP measured prior to glycerin injection was 85.1 ± 41. mv. It decreased to 80.2 ± 4.9 mv 10 min after injection of glycerin. Thereafter the EP gradually recovered and returned to the preinjection level. The EP measured 90 min after injection was 84.2 ± 5.8 mv. The sound evoked responses were also temporarily suppressed shortly after injection of glycerin. An increase of the positive summating potential was always accompanied with a decrease of CM. The CM gradually decreased and loss of CM was 10% 120 min after injection.

b) Ion concentration of the cochlear fluids.

The K^+ concentration in the endolymph increased continuously from 145.8 ± 11.8 mM to 156.9 ± 11.8 mM during the 60 min period after glyceri injection. During this period the K^+ concentration in the perilymph of t scala tympani also increased from 2.9 ± 1.1 mM to 3.8 ± 1.0 mM. The tem- poral changes in the Cl^- concentration in both endolymph and perilymph we found to be similar to those observed in K^+ concentrations of the endolym and perilymph.

c) Osmolarity.

The osmolarity of the blood serum rapidly increased and reached 343.6 ± 4 mOsm/kg 15 min after injection of glycerin. Thereafter the osmolarity of the blood serum decreased gradually and was 308.5 ± 7.2 mOsm/kg 3 hours after injection. The CSF showed the maximum osmolarity (329.0 ± 5.7 mOsm/ 60 min after injection. The osmolarity of the endolymph and perilymph in- creased continuously and 90 min after injection the osmolarity was 340.1 ± 16.6 mOsm/kg in the endolymph and 334.3 ± 15.7 mOsm/kg in the perilymph.

3. Guinea pigs with hypoosmolarity induced by water injection.

a) Cochlear potentials.

No marked changes in the EP was observed. The EP measured 60 min after intraperitoneal injection of water was 89.6 ± 4.5 mV which was comparable to the preinjection EP(89.4 ± 4.6 mV). The CM gradually decreased and reached 87% of the initial CM 60 min after injection.

b) Ion concentration of the cochlear fluids.

The K^+ concentrations in the endolymph and perilymph began to decrease 20 to 30 min after water injection and continued to decrease during the 60 mi observation. The losses of K^+ and Cl^- concentration in the endolymph were 12.8 mM and 4.7 mM respectively. The K^+ concentration gradient between th endolymph and perilymph decreased as a function of time elapsed.

c) Osmolarity.

The osmolarity of the blood serum sampled 7 min after water injection showed loss of 13.6 mOsm/kg. Sixty min after injection its osmolarity de- creased to 260 ± 15.4 mOsm/kg. No recovery was observed during 120 min after injection. The osmolarities of the endolymph and perilymph decrease to 276.3 ± 9.6 mOsm/kg and 273.2 ± 10.0 mOsm/kg respectively 2 hours after water injection.

These findings suggest that the endolymph-perilymph barrier is highly perm able to water and alteration of the blood serum osmolarity tends to decrea the osmotic gradient across the cochlear partition. We plan (1) to study the distribution of glycerin in the cochlear fluids using radiotracer tech niques; (2) to examine possible alteration of water permeability of the

cochlear partition and of water control in the cochlear fluids in noise or drug-treated guinea pigs; (3) to measure the hydrostatic pressure gradient between the endolymph and perilymph under normal and pathologic conditions (hypoosmotic and hyperosmotic).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The mechanisms involved in the kinetics of water in the cochlear fluids have been a subject of extensive studies but convincing data are still limited. These studies are issued as base line data to which water control in the cochlear fluids under pathologic conditions can be compared. This project will make a significant contribution to the Institute Program which provides an essential knowledge base on the impact of environmental factors on human health.

PUBLICATIONS

Konishi, T. and Salt, A.N.: Electrochemical profile for potassium ions across the hair-cell membranes. In Klinke, R. and Hartman, R. (Eds): Hearing-Physiological Bases and Psychophysics. New York, Springer and Verlag, 1983, pp. 39-45.

Konishi, T. and Salt, A.N.: Electrochemical profile for potassium ions across the cochlear hair cell membranes of normal and noise-exposed guinea pigs. Hear. Res. 11: 219-233, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 90030-04 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Toxicants on Membrane-Related Neurochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen C. Bondy	Research Chemist	LBNT	NIEHS
Others:	T. Walsh	Staff Fellow	LBNT	NIEHS
	H. Tilson	Pharmacologist	LBNT	NIEHS
	C. Mitchell	Supv. Pharmacologist	LBNT	NIEHS
	S. Swartzwelder	Staff Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*
U.S. Environmental Protection Agency
Duke University

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neurochemistry

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	0.9	0.9

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project emphasizes the nature of alterations to membranes of the nervous system by organometals for two purposes:

1. To delineate injury to discrete neuronal tracts and to account for observed behavioral changes in terms of damage to specific circuitry. This has been investigated by means of neurotransmitter and neuromodulator receptor analysis. Triethyl lead chloride reduces benzodiazepine binding in the rat hippocampus but not in frontal cortex or striatum. This depression is temporarily correlated with the analgesic effects of triethyl lead. Studies on neonatal rats exposed to triethyl or trimethyl tin also show a specific lowering of hippocampal benzodiazepine binding.

2. To assay for more general damage to cerebral membranes in organometal-treated rats and attempt to describe initial sites of damage caused by these compounds. Studies on membrane-transport systems of trimethyl tin treated animals show regionally distinct metabolic abnormalities. The ability to take up and concentrate amino acids from the plasma is specifically disrupted in the hippocampus while glucose uptake by this region is selectively and transiently elevated. This enhancement may reflect hyperactivity within the region which is ultimately responsible for the known regional morphological damage. Injury to membranes has been looked for by assay of lipid peroxide levels in brain areas and also levels of the protective enzymes superoxide dismutase and glutathione peroxidose. Elevated superoxide dismutase levels, preponderant in the hippocampus implied a reactive response to localized cellular injury.

PHS 6040 (Rev. 1/84) GPO 904-917

PROJECT DESCRIPTION

OBJECTIVES: The goal of this work is the further definition of the mode of neurotoxicity of various organic metal-compounds, especially those known to damage the hippocampus with some selectivity. Neurotransmitter binding studies have been carried out in an attempt to relate known behavioral changes with alterations of activity within various nerve circuits. Another objective has been to account for the neurotoxicity of organometals in terms of damage to various brain membrane systems by peroxidative mechanisms.

It is planned to delineate those characteristics of triethyl lead which resemble inorganic lead and those properties which are distinctively characteristic of triethyl lead. This involves studies of enzymes known to be susceptible to inorganic lead, such as δ-aminolevolinic acid dehydratase and use of agents such as dimethylsulfoxide which may facilitate access of inorganic lead to the brain.

METHODS EMPLOYED: Methods include the preparation of membrane fractions from various regions of the rat brain. These are then incubated together with a radioactive ligand specific for a given binding site and equilibration is allowed to occur. Receptor-ligand complexes are then separated from unbound labelled molecules by filtration. The extent of non-specific interactions is estimated by a parallel incubation in the presence of an excess of a non-radioactive competing chemical.

In addition to routine neurotransmitter analysis, techniques have been used that allow evaluation of calcium binding sites, calcium channels and the sodium pump.

Other laboratory methods include a series of enzyme assays, cerebral uptake of glucose and amino acid uptake and a new method of measuring lipid peroxide levels in the living animal.

MAJOR FINDINGS AND PROPOSED COURSE:

1. Interactions between organometals and biological metals.

 Many calcium and zinc dependent enzymes are unchanged in triethyl lead treated rats (7.9 mg/kg body weight), suggesting no across-the-board competition with such divalent metals. This is in accord with in vitro data showing poor competition between triethylead and calcium-binding sites. However, the zinc and copper dependant enzyme superoxide dismutase is elevated in the hippocampus but not in the striatum or frontal cortex of triethyl lead treated rats. Although in vivo levels of treated rats are unchanged as is the level of the selenium-dependent glutathione peroxidase, this implies an effective defense against superoxide-induced membrane derangement. A degree of hippocampal membrane damage has been found using trimethyl tin, as judged by altered levels of amino acid uptake. These changes are not found in other brain regions and this selectivity is in agreement with the known morphological susceptibility of the hippocampus to organic tin and lead compounds.

2. Biochemical changes relating to behavioral abnormality.

Changes in levels of neurotransmitter and neuromodulator receptor sites have been examined in organometal-treated rats in an attempt to account for distinctive behavioral changes observed. Triethyl lead is known to have a transient analgesic effect and the time course of this was correlated with a region-specific depression of benzodiazepine binding sites. Such a depression was also seen in neonatal rats exposed to organotin compounds. This effect was greater than the depressed hippocampal weight found in trimethyl tin-treated neonatal rats. DNA determinations allowed us to state that surviving hippocampal cells had less benzodiazepine binding sites in these neonatally exposed rats.

The increased seizure susceptibility of triethyl lead exposed animals was found to be reversed by atropine, suggesting the cholinergic system might be involved in this susceptibility. Cholinergic receptors appeared initially depressed and then elevated in the hippocampus of treated rats but more work will be needed to correlate receptor changes and seizure susceptibility on a temporal basis. Depressed receptor levels followed by elevated levels could be explained in terms of an initially hyperactive cholinergic circuit followed by cell death, leading to denervation-related upregulation of postsynaptic receptors. This hyperexcitability followed by neuronal death is thought to be a characteristic effect of excitotoxins on hippocampal tissue. No changes were seen in the receptor levels of the primary excitatory neurotransmitter, glutamate, after triethyl lead treatment.

3. Related studies.

Related studies include the demonstration of the predictive value of a receptor binding survey using a somewhat understood neurotoxic agent; triorthocresyl phosphate. This agent is known to cause cholinergic hyperactivity by inhibition of acetylcholinesterase in the brain. Hens treated with this agent showed a clear decrease in levels of muscarinic cholinergic receptors while dopamine, GABA, adrenergic and serotonin receptors were unchanged. Such a selective decrease gives an immediate clue as to the nerve pathways susceptible to damage by triorthocresyl phosphate, and illustrates the value of the receptor approach.

Another study has shown that glucose uptake and amino acid transport mechanisms in the chick retina are readily modulated by altered sensory input. Thus, these techniques may be used to evaluate regional neuronal activity levels.

4. Future work.

The proposed course of this project will involve new approaches to the question of whether organic metals owe their neurotoxicity largely to their ability to penetrate the blood brain barrier or whether they possess properties fundamentally differing from the corresponding inorganic metals in addition to their ability to pass through membranes.

a. Inorganic lead is not very neurotoxic in adult rats due to its inability to pass into the brain in large amounts. However, we have recently found

that, when administered in dimethylsulfoxide, low doses (25 mg/kg body weight) lead nitrate can cause biochemical and behavioral effects. These effects however do not resemble triethyl lead since the hippocampal benzodiazepine receptor appears elevated rather than depressed and the lead-nitrate-treated rats are hyperalgesic rather than analgesic. We plan to enlarge on these preliminary findings and to determine concentrations of lead in brains of rats exposed to various lead compounds. This work will allow a definition of those properties of organic lead compounds which are completely separate from those of the more prevalent but less neorotoxic inorganic lead compounds.

b. Other preliminary work has shown close parallels between triethyl lead and inorganic lead.

The zinc-dependent enzyme, δ-aminolevulinic acid dehydratase (ALAD) is a key enzyme in heme synthesis and present in many tissues. This enzyme is remarkably sensitive to inorganic lead both in vivo and in vitro, being inhibited significantly at lead concentrations below 10^{-8} M. Several mechanisms have been proposed by which interference with this enzyme can account for much of the neurotoxicity of inorganic lead. We have found that triethyl lead chloride is an equally potent inhibitor of this enzyme both in vivo and in vitro. We are planning a detailed survey of this similarity. This will include various organolead compounds, some of which have rather low toxicity. An initial study suggests a good correlation between the ability of an organic lead compound to inhibit this enzyme and its toxicity. This work will test the validity of the enzymes ALAD and ferrochelatase as potential indices of toxicity of a variety of organic lead compounds.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Triethyl lead is extremely toxic to man (0.25 gm is the estimated toxic dose) although it constitutes a minor fraction of the total lead encountered in the environment. This research will lead to a better understanding of the extent to which organolead toxicity is distinct from that of inorganic lead. The work is concentrated in two areas:

1. Identification of the underlying initial lesion caused by organometals.

2. Accounting for behavioral changes in terms of deranged neuronal circuitry.

The first of these focuses on the primary events of organometal toxicity while the second focuses on the ultimate biochemical changes immediately preceding the expression of abnormal behavior. It is also expected that this work will allow us to define those features which are distinctive to triethyl lead and those which are shared by inorganic lead.

Since around 25% of lead in the brains of normal urban humans is organic, the delineation of organic lead neurotoxicity is of importance.

Expanded knowledge in this area can be of value in diagnosis of various types of organometal poisoning. Evaluation of success of therapeutic measures is

also a potential benefit. Finally, the work may demonstrate the disproportionate hazard posed by some relatively minor components of the total environmental lead burden.

PUBLICATIONS

Ali, S.F., Hong, J.S., Wilson, W.E., Lamb, J.C., Moore, J.A., Mason, G.A$_R$, and Bondy, S.C.: Subchronic dietary exposure of rats to chlordecone (Kepone^R) modifi levels of hypothalamus β-endorphin. Neurotoxicology 3: 119-124, 1983.

Bondy, S.C., Werdel, J., Fletcher, R.T., and Chader, G.J.: Retinal pigment epith lium contains a distinctive strychnine-binding site. Neurochem. Res. 7: 1445-14 1983.

Ali, S.F., Hong, J.S., Uphouse, L.L., Wilson, W.E., and Bondy, S.C.: Effect of acrylamide on neurotransmitter metabolism and neuropeptide levels in several brai regions and upon circulating hormones. Arch. Toxicol. 52: 35-43, 1983.

Bondy, S.C., Ali, S.F., Hong, J.S., Wilson, W.E., Fletcher, T., and Chader, G.: Neurotransmitter-related features of the retinal pigment epithelium. Neurochem. Internatl. 5: 285-290, 1983.

Ali, S.F., Hong, J.S., and Bondy, S.C.: Response of neuropeptides and neurotransmitter binding sites in the retina and brain of the developing chick to reduced visual input. Internat. J. of Develop. Neurosci. 1: 99-103, 1983.

Ali, S.F., Hong, J.S., and Bondy, S.C.: Alterations in retinal neuropeptides of the chick by kainic acid and acrylamide. Brain Res. 274: 115-118, 1983.

Werdel, J.M. and Bondy, S.C.: Uptake of blood-borne materials by the chick retina and brain during visual deprivation. Brain Research (in press) 1984.

Ali, S.F., Abou-Donia, M.B., and Bondy, S.C.: Modulation of avian muscarinic high affinity binding sites by a neurotoxic organophosphate. Neurochem. Pathol. (in press) 1984.

Bondy, S.C. and Hall, D.L.: Modulation of the physiological role of cations in th brain by organic lead compounds. Neurochem. Pathol. (in press) 1984.

Hong, J.S., Hung, C.R., Seth, P.K., Mason, G., and Bondy, S.C.: The result of man ganese treatment on the levels of neurotransmitters, hormones and neuropeptides. Interaction of stress with such effects. Environ. Res. (in press) 1984.

Bondy, S.C.: The biochemical evaluation of neurotoxic damage. Fundamental and Applied Toxicology (accepted) 1984.

Bondy, S.C.: The neurotoxicity of organic and inorganic lead compounds. In Prasad, K.N. and Seth, P.K. (Eds.): Neurotoxic Substances and Their Impact on Human Health. Karger Press (accepted) 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 90031-03 LBNT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assessment of Neurophysiological Effects of Organometals

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: C. L. Mitchell	Supv. Pharmacologist	LBNT	NIEHS
Others: S. Swartzwelder	Staff Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Neurophysiology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The organometals have numerous applications in industrial and occupational settings. The neurotoxicity of these agents, particularly organoleads and tins, is well known. However, their precise sites and mechanisms of action are poorly understood. The purpose of these studies is to characterize the neurophysiological effects of relevant organometals in an attempt to determine the site of action and aid in determining the mechanism of action of selected organometals. We have found that triethyl lead (TEL), but not trimethyl lead, triethyl tin or trimethyl tin markedly increases the sensitivity to pentylenetetrazol induced seizures. This increase in sensitivity is most pronounced in animals receiving multiple pentylenetetrazol injections. There is little, if any, change in sensitivity to a single dose of pentylenetetrazol. Thus, the most striking effect of TEL appears to be an acceleration of the pentylenetetrazol kindling process. This effect of TEL can be attenuated by anticholinergic agents (atropine and scopolamine). Assessment of the neurophysiological and neurochemical bases for pentylenetetrazol-induced kindling and the mechanisms whereby TEL exacerbates it should further our knowledge of excitatory phenomena of the nervous system and how these can be altered by neurotoxic substances.

PROJECT DESCRIPTION

OBJECTIVES: Exposure to many organometals results in a pattern of toxicity resembling that produced by lesions of the limbic forebrain. However, the precise site and mechanisms of action are poorly understood. Moreover, little is known concerning the structure activity relationships of these chemicals.

Our objective is to characterize the neurophysiological effects of organolead and organotin compounds in an attempt to determine anatomical sites of action and aid determining their mechanisms of action. The working hypothesis is that for triethyl lead and trimethyl tin, at least, the limbic system will be perturbed by concentrations of these agents which do not affect other areas of the nervous system.

METHODS EMPLOYED: The initial approach was to determine the single dose, 28 day LD50 by the subcutaneous (SC) route for trimethyl lead (TML), triethyl lead (TEL), trimethyl tin (TMT), and triethyl tin (TET) in Fischer 344 rats. This was done in order to determine a common basis for the selection of doses of those agents in future studies. Subsequently, the ability of 1/4, 1/2, or 3/4 of the LD50 for each of the compounds to exacerbate convulsive activity elicited by pentylenetetrazol (PTZ) was examined. Times after toxicant administration most commonly studied were 1, 7, 14, 21, and 28 days. The purpose of these studies was to establish the doses and time intervals to be utilized for subsequent electrophysiological and neurochemical investigations.

The next step is to investigate the integrity of the limbic system by after-discharge, kindling, and evoked potential techniques utilizing the dosages and time intervals determined from the preceding studies. Comparisons are then made with other systems (e.g., auditory, visual somatosensory) to ascertain the relative specificity of any effect observed. In addition, the ability of anticholinergic and gabanergic agents to alter the effects of the organometals are also investigated.

MAJOR FINDINGS AND PROPOSED COURSE: The effects of TEL, TML, TET and TMT on PTZ-induced seizures have been investigated. PTZ was administered interperitoneally (I.P.) in a dose of 35 mg/kg. This dose reliably produced a mild array of epileptiform signs in the normal Fischer 344 rat. After the animal was treated with PTZ, it was immediately placed in a sound-attenuating chamber and observed for a period of 10 minutes. Any epileptiform signs and their latency were recorded. Two designs have been used to date. The first is a repeated measures design wherein the rats are treated with a range of dosages of any organometal or with the saline vehicle, and then are tested at 1, 7, 14, 21 and 28 days after this acute treatment. This design allows for the assessment of seizure susceptibility and the development of kindling among the rats. The second design is a non-repeated measures one. In these studies the animals are given injections of the metals or saline at 28, 21, 14, 7, or 1 day prior to the single PTZ challenge. Thus, each animal is treated only once with the PTZ and the possible confound of kindling development is controlled.

TEL, but not TML, TMT or TET markedly increases the sensitivity to PTZ. This increase in sensitivity is most pronounced in animals receiving multiple PTZ injections. There is little, if any, change in sensitivity to a single dose of PTZ. Thus, the most striking effect of TEL appears to be an acceleration of the PTZ kindling process. Moreover, anticholinergic agents (atropine and scopolamine) can attenuate this effect of TEL.

Since TEL produced the most striking effect, subsequent studies will concentrate on this compound, using the others where appropriate to determine the specificity of the TEL effects. The following are examples of possible approaches:

1. The neurotoxicity of TEL, TML, TET and TMT on the limbic system will be assessed by investigating their effects on the propensity to (a) lower the threshold for electrically induced afterdischarge in various limbic structures, and (b) to alter the characteristics of evoked potentials.

2. Comparison of intralimbic system effects of microinjection of kainic acid or AF64A (an agent which selectively lesions cholinergic neurons) with similar injections of the organometals using the electrophysiological procedures mentioned above.

3. Use of the in vitro hippocampal slice technique to compare single or multiple unit potentials from selected portions of the hippocampus in treated and control animals. This can be useful both for localizing effects of the organometals within the hippocampus and studying mechanisms. The latter can be done by adding neuropeptides, neurotransmitters or trace metals to the bath to study how these alter the unit potentials.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Organometals are found with great frequency in the environment, yet there is little known about the site(s) and mechanism(s) by which these agents produce their neurotoxicity. The results of this research will provide significant data on where within the brain and how these agents affect neural tissue. This information will be useful in the development and assessment of logical strategies for the detection of toxicity and/or treatment of exposed individuals. In addition it should increase our understanding of the epileptic kindling process and how TEL exacerbates it.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 90033-02 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Milk Bombesin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lawrence H. Lazarus	Research Chemist	LBNT	NIEHS
Others:	W. E. Wilson	Research Chemist	LBNT	NIEHS
	B. H. Irons	Biological Technician	LBNT	NIEHS
	A. Guglietta	Visiting Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*
University of Torino, Italy
University of Rome, Italy
University of Kyoto, Japan
University of North Carolina, Chapel Hill

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Peptide Neurochemistry

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, NC 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new peptide containing immunoreactivity to bombesin was discovered in bovine milk, termed BMB. This peptide chemically differed from both the amphibian peptide and another mammalian bombesin-related peptide, GRP, isolated from porcine non-antral stomach tissue. Preliminary bioactivity studies further determined its differences from existing bombesin peptides, although it belongs to that family of peptides: BMB exhibited a bombesin-specific contraction of isolated rat uterus and guinea-pig large intestine and had a particularly evident effect on rhythmic movement. It was estimated to be 25-40% as active as bombesin and more potent than GRP.

PROJECT DESCRIPTION

METHODS EMPLOYED: The peptide was purified from bovine milk in 500 g batches following acidification, clarification, and neuralization as follows: (a) precipitation in ammonium sulfate, (b) chromatography on a gel filtration column, (c) ODS preparative LC column using stepwise elution with acetonitrile, (d) gradient elution from a semi-preparative ODS column, and (e) analytical HPLC on ODS columns using gradient and isocratic means with acetonitrite and propanol. Pharmacological testing for bioactivity used rat uterus and guinea-pig large intestine in vitro in a physiological medium with continuous recordings of smooth muscle contraction.

MAJOR FINDINGS AND PROPOSED COURSE: The isolation scheme developed during the first part of FY-84 resulted in an apparently homogeneous absorbance peak that coincided with immunoreactivity. However, that initial attempt did not yield sufficient quantities for structural analyses, although determination of its bioactivity indicated that it was a new peptide which differed from both amphibian bombesin and GRP.

The proposed course of research will increase the material processed in the isolation procedure in order to obtain 10-20 μg of peptide suitable for chemical analyses and to further study its bioactivity in in vitro and in vivo. Monoclonal antibodies will be produced in order to have sufficient quantities for studies on its distribution in other mammalian milk, mammary tissue and their tumors, and neuronal tissue. The source of BMB remains unresolved and the tissue of origin is unknown.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The discovery of a new peptide in bovine milk that potentially affects the regulation of numerous gastrointestinal hormones and gastric acid production in the stomach is very interesting in light of the common practice of using milk in the treatment of ulcers. If analogues of this peptide could be synthesized, it may function in a natural (i.e., non-drug) mode to alleviate stomach ulcers without an effect on GI homone levels. Since other mammalian forms of bombesin are found in neuronal tissue, its presence with specific classes of nerve fibers or brain regions may further enlighten studies in which amphibian bombesin affects saiety, glucose levels and other metabolic processes under neuronal regulation as well as behavior.

PUBLICATIONS

Jahnke, G.D. and Lazarus, L.H.: A bombesin immunoreactive peptide in milk. Proc. Natl. Acad. Sci. 80: 578-582, 1984.

Gaudino, G., Lazarus, L.H., Wilson, W.E., and Tully, M.: Does human milk contain bombesin-like material? In Peeters, H. (Ed.): Proteins of the Biological Fluids. London, Pergamon Press, 1984.

Gaudino, G., De Bortoli, M., and Lazarus, L.H.: A Bombesin-related peptide in experimental mammary tumors in rats. Internatl. Cong. Endocrin. of the Breast, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 90034-01 LBNT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Rabbit Stomach Peptide [Physalaemin-like Material (PLIM)] in Mammalian Tissue

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William E. Wilson	Research Chemist	LBNT	NIEHS
Others:	L. H. Lazarus	Research Chemist	LBNT	NIEHS
	B. J. Irons	Biological Technician	LBNT	NIEHS
	D. Harvan	Chemist	LMB	NIEHS
	A. Guglietta	Visiting Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*

University of Kyoto, Japan
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University of Rome, Rome, Italy

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SECTION

Peptide Neurochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The long-term objectives of this research project are to identify and characterize this new peptide in neuronal tissue and to determine its physiological and pharmacological mode of action. This material was originally isolated as an immunoreactive substance that cross-reacted with a physalaemin-specific antiserum; mass spec date gave a mass ion of 866 and amino acid analysis indicated that it was an octapeptide. This peptide is not a tachykinin due to the absence of amino acids that typify that class of bioactive peptides. Preliminary data on its biological activities indicate an increase in tonus and phasic motility of guinea-pig ileal smooth muscle, with slower onset and relaxation than physalaemin. In rabbit large intestine, it appeared to inhibit tone and motility which was reversed by physalaemin. Thus, this peptide may prove to be a prototype of a new class of mammalian peptide hormones.

This was formally Project No. Z01 ES 25019-03.

PHS 6040 (Rev. 1/84) GPO 904-917

PROJECT DESCRIPTION

METHODS EMPLOYED: The peptide was purified using the following scheme: (a) ethanol-formic acid extraction of lyophilized rabbit stomachs, (b) removal of ethanol and concentration, (c) passage through strong anion and cation exchange resins, (d) C-8 reverse phase LC using stepwise elution with ethanol, (e) HPLC on silica gel, and (f) HPLC on ODS and diphenyl reverse-phase columns. Pharmacological monitoring of bioactivity used guinea-pig ileum and rabbit large intestine *in vitro* in a physiological medium with continuous recordings of smooth muscle action.

MAJOR FINDINGS AND PROPOSED COURSE: The purification scheme developed over the past year resulted in a highly purified sample. Mass spec analysis yielded a mass ion of 866 for the peptide. The amino acid composition indicated that it was an octapeptide containing the following amino acid residues: 2 glutamic acid, 2 aspartic acid, and 1 each of proline alanine, valine and isoleucine. Ammonia accounted for the remaining mass. The precise sequence was not yet established, although based on antibody specificity, a portion of the sequence should be Asp-Pro-Asn.

The proposed course of research concerns the distribution of this new peptide in neuronal tissue and the determination of its bioactivity. The production of new antisera generated against this peptide and the subsequent development of a radioimmunoassay will assist in future studies; e.g., the formation of immunoaffinity columns to be used in purifying the peptide from nerve tissue, and the direct localization and distribution in tissues by immunohistochemistry coupled with a RIA. The pharmacological and physiological assessment *in vitro* and *in vivo* of its bioactivity will further delineate its unique chemical properties.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The discovery and isolation of a new neuropeptide from mammalian tissue is a remarkable find. That structural homology exists between this peptide and an amphibian peptide is unique which suggests an evolutionary conserved peptide sequence. Since this peptide was found in respiratory tissue, nerves, spinal cord, and throughout the gastrointestinal tract (including Brunner's gland), this peptide could conceivable be involved in multiple physiological roles and may lead to clues on cystic fibrosis or other diseases that affect salt and water balance. Its association in neuronal tissue could further implicate this peptide in a behavioral role and pave the way for investigations with neurotoxicants.

PUBLICATIONS

Wilson, W.E., Irons, B.J., Carlton, D.L., Lazarus, L.H., and Erspamer, V.: Physalaemin-like immunoreactive material (PLIM) from rabbit stomach: partial purification and characterization. Endocrine, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 90035-01 LBNT

PERIOD COVERED: October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Use of Ornithine Decarboxylase in the Detection of Tissue Activation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Stephen C. Bondy	Research Chemist	LBNT	NIEHS
Others: J.-S. Hong	Pharmacologist	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neurochemistry

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, NC

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to exploit the rapid changes in levels of ornithine decarboxylase (ODC) in tissues that respond to damage by regenerative or adaptive changes.

Chlordecone administration to rats, at levels causing tremor (40 mg/kg body weight) causes a 21-24-fold increase in levels of adrenal ODC. This is a rapidly occuring, reversible event of much greater magnitude than any other biochemical response to chlordecone hitherto reported. Other neurotoxic agents, such as triethyl lead chloride also cause a several-fold elevation in the adrenal level of this enzyme.

These data will be expanded so that the precise region of this effect will be determined together with a definition of those neuronal or endocrine inputs which are needed to allow this increase. A time course study allows the detection of those cerebral or peripheral areas which initially respond to the presence of various neurotoxicants.

The significance of this project is that sites of reaction to extremely low levels of toxicants may be detected by this means.

PHS 6040 (Rev. 1/84) GPO 904-917

PROJECT DESCRIPTION

METHODS EMPLOYED: The main technique employed is the assay of ODC activity by trapping of $^{14}CO_2$ evolved during the decarboxylation of labeled ornithine. The instability of this enzyme and possibility of non-specific decarboxylation have to be taken into account.

MAJOR FINDINGS AND PROPOSED COURSE: ODC is an enzyme involved in polyamine systhesis. Elevated levels of this enzyme imply high levels of cellular activity. Such activity may include proliferation, hypertrophy, secretion, growth and regeneration. The short half life (20 min) of this enzyme allows rapid detection of the onset of any of the above processes.

OBJECTIVES: Two main objectives exist:

1. The detection of the primary site of action of organochlorines known to disrupt the hypothalamic-adrenal axis and definition of the components necessary for the expression of neurotoxicity.

2. The demonstration of the potential applicability of ornithine decarboxylase as a marker for abrupt neuroendocrine changes in response to environmental or chemical factors. The precise locus of commencement of a series of adaptive modifications may be located in this manner.

This enzyme is known to be elevated in the adrenals of animals treated with a tremorigenic agent, oxotremorine. The effect of the tremorigenic organochlorine, chlordecone on this adrenal enzyme was examined. Within 4 hours of administration, a massive increase in ODC was observed and this change persisted at a lower level for 4 days. Before reaching control levels in a week another insecticide of this class mirex, also elevated adrenal ODC levels as did triethyl lead chloride. Although the increase in this latter case was only two-fold, the effect persisted over a week. Brain levels of ODC in treated rats will soon be examined.

It is planned to determine whether the observed effect of chlordecone:

a) is primarily in the adrenal cortex or medulla

b) is reproducible in hypophysectomized animals

c) is reproducible in splanchnectomized animals

d) can be brought about by non-tremorigenic organochlorines such as o,p'-DDT

e) also exists in the hypothalamus and other brain areas.

Together with a detailed time course study, these data should allow a determination of the primary target of organochlorines, and which effects can be considered secondary.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This work will throw light on potential areas which are those first affected by organochlorines and may allow identification of many known effects as indirect. A distinction between tremorigenic and non-tremorigenic organochlorines may lead to definition of more than one type of primary impact site.

The work also illustrates the potential of ODC as an index of tissue activity and thus a means of detection of focal sites of toxic damage. This enzyme, much exploited in cancer research, has great largely unexplored value in studies of damage to nerve tissue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 90036-01 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Animal Model of Organometal Neurotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hugh A. Tilson	Pharmacologist	LBNT	NIEHS
Others:	T. J. Walsh	Staff Fellow	LBNT	NIEHS
	J. S. Hong	Pharmacologist	LBNT	NIEHS
	R. L. McLamb	Technician	LBNT	NIEHS
	D. Schulz	Graduate Student	LBNT	NIEHS
	J. Chrobak	Graduate Student	LBNT	NIEHS
	S. Bondy	Research Chemist	LBNT	NIEHS

COOPERATING UNITS *(if any)*
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SECTION
Neurobehavioral

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Organometal (OM) compounds are used for a variety of industrial applications and their trialkyl derivatives produce both neural and behavioral toxicity. The goals of this research are to characterize the time and dose-related neurotoxicity of OMs, examine the potential neurobiological substrates of these effects, and study their significance relative to the CNS. The behavior of animals exposed to OMs is similar to that of animals having limbic forebrain lesions. Pharmacological studies implicate catecholaminergic systems in some of the behavioral effects of triethyl lead. Subsequent studies have examined the effects of OMs on limbic system function. The limbic system modulates behavioral reactivity, emotionality and memory and appears to be very sensitive to many toxicants, including OMs. The effects of OMs have been compared to specific cytotoxicants such as AF64-A, 6-OHDA, and DSP-4. Studies to date indicate that the behavioral effects commonly associated with limbic damage appear to be related to a disruption of cholinergic function. These studies suggest that OMs and other neurotoxicants may be useful in the study of the neurobiological basis of some types of metabolic encephalopathies and degenerative disorders.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

1. Nature of the Problem: Organic lead (OL) compounds are used for a variety of industrial applications. Tetraalkyl leads serve as the major antiknock additives in fuel products and their trialkyl metabolites produce both behavioral and neurological toxicity. Despite the environmental impact of OLs and the continuing reports of human poisoning the neurotoxicity of these compounds has not been systematically investigated.

2. Objectives: The purpose of this research is to: (1) characterize and study in an animal model the neurobehavioral toxicity of representative organometal (OM) compounds, (2) identify possible behavioral and neural mechanisms of action responsible for these effects, and (3) study their significance with regard to central nervous system function.

3. Experimental Approach: Procedures developed in this laboratory are used to determine time- and dose-related neurobehavioral effects of representative agents. Once effects have been characterized, subsequent studies are designed to associate behavioral and neurological effects with neurochemical, neurophysiological or neuromorphological alterations.

4. Recent Accomplishments and Significance:

a. Time- and Dose-Response Comparisons.

Initial attempts to quantify the neurobehavioral effects of triethyl lead found that repeated injection of 1 to 2.5 mg/kg per day for 5 days resulted in dose- and time-related change in responsiveness to noxious and nonnoxious stimuli. Improvement of two-way shuttle box avoidance and impaired passive avoidance suggested limbic system involvement. Subsequent experiments found that TEL-induced alterations in reactivity to noxious stimuli were probably mediated at the level of the central nervous system and appear to be associated with changes in transmitters and neuromoduators involved in the control or modulation of emotional reactivity to environmental stimulation.

Subsequent experiments sought to systematically determine the effects of TEL and related OMs following acute administration. These studies compared the dose (1/4, 1/2, 3/4 the acute LD50) and time-related effects of triethyl lead (TEL) and trimethyl lead (TML) on motor activity, neuromotor performance, response to acoustic stimuli and hot-plate latencies. TEL increased hot-plate latencies and locomotor activity without affecting neuromotor performance. In contrast, TML altered neuromotor function (increased fore and hind limb grip-strength) and increased hot-plate latencies 21 and 28 days after dosing. These data demonstrated that TEL and TML produce different profiles of behavioral toxicity. Collaborative studies found that TEL produced significant cellular swelling in the hippocampal granule neurons as well as hypertrophy and accumulation of mitochondria in many dorsal root ganglion neurons. Unlike TEL, TML induced only minimal neuromorphological changes which were confined to the dorsal root ganglia.

To examine whether different OMs would produce similar neurotoxic effects, we compared the dose- and time-dependent effects of trialkyl lead (TEL & TML) and tin (TET & TMT) compounds on hot-plate latencies. All four compounds increased response times on the hot-plate, but the onset and duration of these effects varied. For example, TEL and TET increased latencies 1, 7, 14 and 21 days after dosing while TMT increased it throughout the 28 day test period and TML produced a late-developing antinociception. Thus, antinociception is a common, and perhaps clinically important, consequence of OM exposure.

Neurochemical studies comparing the OMs found little consistent effect on neurotransmitters and neuropeptides in various regions of the CNS. However, TMT decreased putative inhibitory transmitters glycine, taurine, and GABA in the hippocampus and frontal cortex at a time of peak behavioral effect. Regionwide increases in glutamine suggested that TMT alters the disposition of glutamine and/or glutamate or that it increases ammonia content in the brain. TML, TET and TEL had no effect on any of the amino acids in any of the brain regions that were tested.

b. Mechanisms of TEL-Induced Antinoceception.

Since TEL-induced antinociception has been found to be a robust phenomenon, we examined behavioral and biochemical processes which might contribute to its expression. Rats dosed with 3/4 LD50 of TEL and tested 7 days later on the hot-plate exhibit hot-plate latencies which are twice as long as those of the controls. The topography of TEL-treated rats on the hot-plate was distinctly different from that observed in animals treated with an equianalgesic dose of morphine. The morphine-treated rats were lethargic and hypokinetic while the TEL group exhibited increased activity and apparent escape behavior when placed upon the hot-plate. Amphetamine also increased hot-plate latencies and produced a pattern of behavioral effects similar to that observed in the TEL group. Repeated exposure to a non-functional hot-plate as well as repeated testing attenuated, but did not reverse, the TEL antinociception suggesting that reactivity to the novel test environment contributes to the increased hot-plate latencies.

Subsequent studies examined the potential involvement of brain transmitters in TEL-induced antinociception. Rats were pretreated with TEL or vehicle and challenged prior to testing with compounds that selectively interact (agonists or antagonists) with biogenic amine or neuropeptide systems. If a given transmitter system is uniquely involved in a behavioral effect (i.e., analgesia) then pharmacologically augmenting or decreasing its activity should alter the expression of that behavioral effect. These studies revealed that cholinergic and serotonergic antagonists had no effect on TEL-antinociception. Naloxone attenuated the increased latencies in a dose-dependent manner but morphine was found to be additive with TEL suggesting that endogenous opiates might play some role in the expression of this effect.

Chlordiazepoxide (CDZ), a benzodiazepine (BDZ), did not alter the increased hot-plate latencies. A role for BDZs in TEL antinociception however is suggested by two observations; first CDZ can attenuate the analgesic effects of short-term repeated exposure to TEL and secondly we observed a negative correlation between

hot-plate latencies and flunitrazepam binding in the hippocampus following TEL. BDZ receptors were decreased in the HPC only at the same time points when hot-plate latencies were increased. BDZ involvement in reactivity to aversive stimuli in general is supported by the observation that RO15, 1788, a selective BDZ antatonist, produced a time and dose-related increase in hot-plate latencies which was not reversible by naloxone.

c. Involvement of Brain Dopamine Systems in TEL Neurotoxicity.

Pharmacological challenge studies found that DA agonists enhanced TEL antinociception while depletion of brain DA with 6-OHDA completely blocked it. These studies suggest that brain DA may be preferentially involved in TEL neurotoxicity. Subsequent studies evaluating the responsiveness of TEL-treated rats to DA agonists further supported this hypothesis. Rats pretreated 7 days prior to testing with TEL exhibited a shift to the left in the dose-response curve for apomorphine and amphetamine-induced hypermotility. This enhanced behavioral response to DA agonists was not secondary to (1) weight loss, (2) alterations in amphetamine's time course of action or (3) accumulation or distribution of radioactive amphetamine in the brain. Collaborative biochemical studies revealed that TEL exposure enhanced DA- (but not norepinephrine) stimulated adenylate cyclase in the olfactory tubercles and increased the number of DA (but not serotonin) binding sites in the striatum. Thus, acute exposure to TEL enhances the biochemical and behavioral response to both pharmacological and environmental stressors.

d. Effects of TEL on Cognitive Behavior.

The effect of TEL on learning and memory was evaluated in a radial-arm maze (RAM) task. Rats were dosed with distilled water, or either 1/2 or 3/4 the acute LD50 dose of TEL and trained 21 days later in a RAM. TEL did not alter the rate of acquisition of the task over 20 daily test sessions. To increase the complexity of the task each rat was removed from the maze following his fourth choice; he was returned to the maze 2 hours later to complete the task (i.e., obtained the remaining pellets). These delay sessions continued for 10 trials (one per day) and again the performance of the TEL group did not differ from that of the controls. All groups retrieved the pellets from the previously unentered arms and avoided the arms where food had been obtained prior to the delay. These studies demonstrated that at the doses employed TEL did not alter learning or memory as assessed by the RAM.

e. Effects on Limbic Forebrain Function.

Several laboratories have indicated that OMs such as trimethyltin may have selective effects on limbic forebrain structure and function. As part of the current research program, various neurotoxicants, including organometal compounds, are being used to study the neurobiological role of the hippocampus and related structures. One approach to this problem is to examine the behavioral consequences of destroying neurotransmitter pathways innervating limbic structures. Initial studies infused AF64A, a cholinergic neurotoxicant, into the lateral ventricles which produced transient and time-related increases in motor activity and decreases in hot-plate latencies. Retention of a step-through passive

avoidance (PA) task was also impaired. Performance in a radial-arm maze (RAM) was impaired 60-80 days after treatment. The AF64A groups made fewer correct responses in their first 8 choices and required more total selections to complete the task and exhibited an altered pattern of spatial responding in the maze. These behavioral effects were associated with a 50% decrease of acetylcholine (ACh) content in the HPC and the frontal cortex. Regional concentrations of DA, NE, 5-HT their metabolites, and Ch were not affected.

The cognitive impairments produced by AF64A suggested that the loss of cholinergic input to the HPC was responsible for these deficits. The anatomical specificity of these effects was determined by injecting AF64A directly into the dorsal HPC, the corpus striatum or the nucleus basalis. These studies revealed site-specific behavioral alterations.

Destroying one of the primary targets of the cholinergic septohippocampal afferents (i.e., granule cells in the dentate gyrus) with colchicine also impairs cognitive function. Intradentate colchicine increased motor activity without affecting hot-plate latencies or the acoustic startle response. Retention of a PA response and performance in the RAM are also impaired following colchicine. Colchicine treated rats were also more sensitive to the motor stimulant effects of scopolamine, but not amphetamine. These data suggest that intradentate colchicine impairs cognitive processes and alters cholinergic dynamics in the HPC. These data, together with the aforementioned AF64A studies, suggest that cholinergic processes in the HPC play an important role in modulating behavioral reactivity and cognitive function. Collaborative studies are evaluating the specificity of the colchicine-induced damage to the granule cells and the impact of this damage on cholinergic cell bodies in the medial septum and the diagonal band.

B. PLANS FOR THE SUBSEQUENT YEAR:

Collaborative studies will examine the relationship between tissue and blood levels of OLs and their behavioral effects. This information is necessary to interpret the behavioral effects observed thus far with these chemicals. For example, the effect of TEL on brain DA systems might be due to a preferential accumulation of toxicant in DA cell bodies or terminal fields.

Other collaborative studies will assess whether TEL-treated rats exhibit an enhanced biochemical response (i.e., increased DA turnover) to pharmacological and environmental stressors. Secondly, if the alterations in DA receptor number and adenylate cyclase activity (i.e., supersensitivity) are responsible for the enhanced behavioral response to dopaminomimetics, then infusing DA directly into dopaminergic terminal fields (i.e., the corpus striatum or nucleus accumbens) should also elicit an augmented response in the TEL group. This would further elucidate the biochemical and anatomical specificity of TEL's neurotoxic effects.

Future experiments will also examine the nature and reversibility of the cognitive deficits induced by AF64A and colchicine. Studies are now in progress to determine the effects of these compounds on reference memory (i.e., learning of a rule that is relevant for all trials) and working memory (information that

is useful for only 1 trial). A delayed match to non-sample task with delays of various length imposed between choices 4 and 5-8 in the RAM will also be utilized to assess mnenomic ability. These studies should further elucidate the involvement of HPC cholinergic processes in cognitive function.

Other experiments will examine whether the cognitive impairments induced by AF64A and colchicine can be reversed or attenuated by pharmacological manipulations. For example, if the cognitive deficits induced by AF64A are due to a loss of cholinergic tone then cholinomimetics such as anticholinesterases, muscarinic agonists, compounds that facilitate high affinity choline uptake or neuropeptides which modulate ACh turnover might reverse these deficits.

Secondly, there is evidence that opiates impair memory processes and oppose ACH-mediated transmission. Since AF64A-treated rats are more sensitive to the analgesic effects of morphine it is conceivable that an inhibitory influence of opiates on an already compromised cholinergic system might contribute to the observed cognitive deficits. If this is the case then opiate antagonists such as naloxone might attenuate these deficits. Collaborative studies will further examine opiate involvement in learning and memory by evaluating whether release of endogenous opiates (and perhaps their influence on cholinergic processes) contributes to the amnesia induced by electroconvulsive shock.

Finally, additional studies will be performed which will study in greater detail the putative role of the HPC as a comparator in the regulation of behaviors such as matching and melioration on concurrent food reinforced schedules. In addition, behavior maintained on a food reinforced irt> 10 sec schedule will be used to quantify the role of the HPC in the control of inhibitory responding. Pharmacological challenges will be used to assess potential alterations in the sensitivity of various neurotransmitter or neuromodulator systems.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This research will provide information concerning the characterization and elucidation of mechanisms responsible for organometal neurotoxicity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 90037-01 LBNT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxicological Perturbations of Behavioral and Neural Development

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Behavioral and Neurological Toxicology

SECTION

Neurobehavioral

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The development of the central nervous system and its functional output, behavior, reflect a series of maturational events that are highly ordered and precisely timed; the consequences of early disruption can be acute and severe or more subtle and insidious.

Neonatal exposure to chlordecone, a prototypic organochlorine, produced tremor and increased reflex reactivity in preweaning rodents, effects similar to those observed in acute toxicity studies with adult animals. Sex-dependent body weight changes persisting into adulthood strongly implicated alterations(s) in the sexual differentiation of hypothalamic centers involved in feeding and weight regulation. Long-term alterations in brain catecholaminergic and serotonergic function were suggested by specific pharmacological challenges. Deficits in retention of learned responses dissociable from nonspecific alterations, such as hyperactivity, were also uncovered. Both short- (hypersecretion) and long-term (depression in basal levels) alterations in circulating and adrenal steroids indicated that chlordecone-induced changes in behavioral reactivity and the modulation of memory were produced, at least in part, by acting on the adrenal gland and the feedback regulation of the hypothalamic-pituitary-adrenal axis.

Neonatal exposure to triethyl lead (TEL), a representative organometal, was shown to permanently affect behavioral processes in a manner similar to that observed after damage to the septal-hippocampal system. Long-term behavioral alterations were independent of sensory modality and represented a hyperreactive response of the animal to the test environment. Preferential and permanent destruction of hippocampal pyramidal cell fields (regio inferior) were observed under light microscopy. Pharmacological probes suggested long-term alterations in cholinergic, but not dopaminergic, function.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

1. Nature of the Problem: The development of the central nervous system (CNS) and its functional output, behavior, reflects a series of maturational events that are highly ordered and precisely timed. Disruption of these processes can have severe consequences. On the other hand, the developing animal also displays a great capacity for plasticity and the interplay between these factors determines the net functional outcome for the organism. The maturing organism presents many opportunities to study long-term consequences of early perturbation, examine mechanisms underlying such alterations, and elucidate mechanisms and processes controlling behavioral and neural development.

2. Objectives: The purpose of our research is to: (1) to characterize the developmental neurotoxicology following exposure to two different classes of environmentally relevant agents (organochlorines and organometals), (2) study the mechanisms by which behavioral and neural toxicity is expressed, and (3) further examine the development of a specific behavioral and neural process implicated in initial studies. The ultimate aim of our research is to derive working models for predicting the long-term consequences of early insult to the CNS.

3. Experimental Approach: Procedures developed in this laboratory are used to determine dose- and age-response alterations in the ontogeny of behavioral and neural development. Measures of motor and reflex development, habituation and learning/memory are utilized. Environmental manipulations and pharmacological agents are employed to probe the functional integrity of potential mechanisms and sites of action. Neuroendocrine and neuromorphological studies are employed to assess more molecular aspects of selected behavioral and/or neural dysfunction.

4. Recent Accomplishments and Significance:

a. Neonatal Organochlorines.

Characterization and potential mechanisms of chlordecone. Initial experiments found few effects on behavioral and/or neural function in post-weanling and adults animals with a routine screening test battery, although subtle long-term alteration in the performance of a food-reinforced operant discrimination task suggested the possibility of residual effects. Subsequent experiments assessed more directly the clinical signs observed following chlordecone exposure (i.e., tremor, hyperreactivity and altered growth rate) and a developmental time course for these effects was obtained. Initial studies also indicated that the vehicle, dimethylsulfoxide, had no detectable effects on these measures. These later experiments revealed a significant sex-dependent effect on body weight which persisted into adulthood. This effect is similar to that observed following neonatal treatment with estrogen, suggesting that chlordecone may have had an organizational effect on neural (hypothalamic) centers involved in feeding and weight regulation. Although tremor and hyperexcitability were quantified in weanling animals, there was no indication of residual effects when assessed at

adulthood. However, long-term alterations in catecholaminergic and serotonergic pathways were observed following pharmacological challenges.

Two separate series of experiments examined the effects of developmental exposure to chlordecone on the retention of a conditioned response using paradigms that permitted the assessment of specific "associative" effects independent of generalized deficits in performance (i.e., due to differential sensitivity to footshock, hyperreactivity to the conditioned stimuli, differences in motor activity). Retention of a passive avoidance response was impaired by early chlordecone exposure using a 7-day, but not a 1-day, retention interval. A second experiment found that prior acquisition of a one-way avoidance task altered the directional bias usually observed during subsequent acquisition of a two-way avoidance task, suggesting a retention impairment for a specific attribute of the original one-way avoidance training. In both studies, chlordecone-exposed animals were found to have significantly higher levels of plasma corticosterone than controls after the retention test. Although the alterations in hormonal status during preweaning and immediate post-weaning development may reflect direct pharmacological effects of chlordecone, long-term decreases in basal levels of circulating and adrenal corticosteroids and adrenal hyperplasia have been observed. Collectively, these findings suggest that chlordecone-induced changes in behavioral reactivity and the modulation of memory may be produced, at least in part, by acting on the adrenal gland and the feedback regulation of the hypothlamic-pituitary-adrenal axis (HPA).

Another component of this research program concerns our efforts to control for and in some cases assess the "nonspecific toxicity" of early exposure to environmental agents. Nonspecific toxicity may be attributable to such conditions as mild undernutrition or early stress which undoubtedly accompany exposure to nearly all neurotoxic compounds. Because either of these conditions alone may produce alterations in behavioral and neural function which persists into adulthood, we have systematically manipulated several variables (i.e., as the influence of early behavioral testing, the vehicle, and the age at which toxicant exposure occurs. Although these experiments are not yet complete, it is evident that behavioral and/or neural toxicity is affected by subtle environmental and procedural variables.

b. Neonatal Organometals:

Characterization and mechanisms of triethyl lead toxicity. The behavioral and/or neural toxicity of early triethyl lead exposure was characterized for short and long-term sequelae. At 5 days of age one pup of each sex per litter was administered a s.c. injection (20 ul) of either distilled water, 4,5- or 9.0-mg/kg TEL. The fourth pup of each litter was an undernourished control animal allowing for the comparison of non-specific and specific effects of TEL. The undernourished pups were weight-matched throughout development to the 9.0 mg/kg TEL-animals by placement with a nipple-ligated surrogate dam on postnatal days 5, 7, 10, and 14. Preweaning behavioral assessments indicated that relative to both control groups, TEL-toxicity produced a biphasic dose related response. An initial period of excitability, indicated by the presence of tremor, occurred during the second week of lite. This phase was followed by one of reduced activity and auditory startle measurements. At day 21 (weaning) animals

were sacrificed for quantitative morphometric and brain weight assessments. Weanling brain weights were depressed by neonatal TEL-exposure, an indicator of nonspecific toxicity. Morphometric analyses revealed a specific loss of hippocampal regio inferior pyramidal cells without evidence for widespread neural damage.

Further studies explored potential long-term alterations and/or functional recovery following neonatal TEL-exposure. Adult rats exposed neonatally to TEL displayed a hyperreactive response in a number of behavioral tasks, as indicated by a lack of habituation to activity testing, immobility during spectral analysis profile of body movement, and increased auditory startle responsiveness. Further examination of the learning abilities of TEL-treated animals using a two-way active avoidance task which revealed no deficit; however, special control groups indicated that the TEL-treated animals were hyperresponsive to the testing situation. The hyperreactivity was not attributable to an increase in pain sensitivity, as indicated by subsequent shock reactivity and hot-plate tests. In each of these tasks, TEL-effects were detected in the absence of, or were greater than, effects of early undernutrition. Analyses of regional brain weights at 140 days of age indicated that only the hippocampus was depressed in wet weight in both sexes. Quantitative morphometry further indicated the selective cell loss in hippocampal areas CA3a and CA3b of TEL-treated animals was of a permanent nature. Thus, neonatal TEL-exposure produced long-term hyperreactivity independent of early undernourishment effects and sensory modality. These data suggest that the behavioral and neuromorphological effects of TEL are due to preferential damage to the hippocampus.

Hippocampal lesions have been found to produce alterations in a number of brain regions, particularly in the dopaminergic basal ganglia systems and the cholinergic septal-hippocampal pathway. As neonatal TEL-exposure was found to produce relatively selective hippocampal damage, we examined the functional status of dopaminergic and cholinergic systems as an index of secondary effects resulting from TEL-exposure. Offspring of Fischer-344 dams were dosed on postnatal day 5 and undernourished control animals produced, as described for the previous neonatal TEL studies. In agreement with our previous findings, neonatal exposure to TEL produced long-term alterations in behavioral reactivity. However, the hyperreactivity did not appear to be modulated by dopaminergic systems, but was modulated by cholinergic manipulations in a gender-specific and task-specific manner.

B. PLANS FOR THE SUBSEQUENT YEAR

1. Studies on the effects of developmental chlordecone exposure. Future experiments will investigate the effects of chlordecone on neural systems known to mediate and/or modulate behaviors altered by neonatal chlordecone exposure. The developmental time course for the onset of tremor will be investigated in greater detail and compared to that of pharmacological agents having tremorigenic activity. Because earlier research suggested that chlordecone has significant effects on the disposition of brain serotonin, we propose to investigate the role of the inhibitory serotonergic system on the climbing fibers to the cerebellum. The postnatal ontological pattern of cerebellar development

suggests that this structure may be differentially vulnerable. Studies concerning the ontological time-course of chlordecone-induced tremor will be followed by similar studies concerning other effects (exaggerated startle response, altered habituation) of this agent.

Future research will attempt to determine the contribution of nonassociative factors to the effects of neonatal chlordecone on memory. This work will continue to rely on multivariate statistical approaches to the assessment of selective retention deficits. Although the memory deficits measured thus far may be specific to a single dimension of task performance, the possibility still exists that there is a subtle alteration in task acquisition underlying the retention deficit. For example, toxicant-induced overshadowing or blocking might have occurred to one or more of the salient stimulus components of the task. Because it is impossible in developmental paradigms to train the animal prior to toxicant exposure, older animals must be used in these same paradigms in order to preclude any differences in task acquisition as a causal factor for the retention deficits. Thus, adult animals will be trained 2 weeks after toxicant exposure in various avoidance tasks and retention assessed at a later time.

2. Investigation of early hormonal imbalances and their consequences for behavioral and neural development. Our previous work indicates that alterations in hypothalamic-pituitary-adrenal function (HPA) may be responsible for some of the long-term changes in behavioral and neural function that follow developmental exposure to chlordecone. Future studies will identify and study the role of alterations of the HPA in organochlorine toxicity, and also in relation to the more basic question of organizational effects on behavioral and neural development.

Initial studies will measure the distribution and half-life of radiolabelled chlordecone in order to determine if the adrenal gland is a major distribution site in the neonate and the extent to which chlordecone may have a primary or direct effect on the CNS. Subsequent studies will determine the effect of neonatal exposure to chlordecone on basal levels of corticosterone and ACTH using radioimmunoassays (RIAs). The influence of neonatal chlordecone on the functional integrity of the HPA axis will then be determined by testing adrenal (ACTH injection) and pituitary reserve (metyrapone). The role of early hormonal imbalance in the long-term consequences for behavioral and neural development will be investigated using exogenous administration of various adrenal steroids.

In another series of studies, the effects of chlordecone will be compared to those of o,p´-DDT and p,p´-DDT, two organochlorine insecticides that have similar neurohormonal effects as chlordecone. However, the o,p´-DDT isomer has little neurotoxicity and has greater steroidal activity than the p,p´-DDT form. To the extent that hormonal alterations induced by neonatal chlordecone underlie the neurobehavioral and/or functional toxicity of the HPA axis, it is expected that the effects of o,p´-DDT isomer will more closely resemble those of chlordecone.

3. Further characterization of triethyl lead toxicity. Neurotoxicants have been widely used in recent years to study both structural and functional aspects of the central nervous system. However, their use in developmental investigations has been limited by the lack of preferential agents. Future studies using neonatal TEL-exposures may yield information not only about the site and mechanisms of TEL-toxicity, but TEL may also become a useful neurobiological tool for exploring behavioral and neural development.

Our plans are to investigate with specific pharmacological agents the putative excitatory and inhibitory neural circuits suspected of being responsible for the hippocampal pyramidal cell damage. Our major goal is to determine whether, following protection of the pyramidal cells from neuronal excitation with gabergic agonists and glutaminergic antagonists, TEL has a cytotoxic effect. If the pyramidal cells can be protected from any direct TEL induced damage, then cell death must be attributable to the pharmacological overexcitation of this pathway. Collaborative studies to examine potential neuronal rearrangements underlying either recovery of behavioral function or a failure to observe such recovery. Given a working mechanism for the behavioral and neural toxicity of TEL, it will then be of interest to assess the generality of this model with agents suspected of having similar effects (e.g., kainic acid, trimethyl lead).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This research will provide information concerning the effects of environmental agents on the developing organism.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 90038-01 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Animal Model of Organochlorine Neurotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hugh A. Tilson	Pharmacologist	LBNT	NIEHS
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	T. J. Walsh	Staff Fellow	LBNT	NIEHS
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	D. Herr	Guest Worker	LBNT	NIEHS
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	J. Peterson	Technician	LBNT	NIEHS

COOPERATING UNITS *(if any)*

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LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neurobehavioral

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Organochlorine chemicals such as chlordecone, p,p′-DDT and lindane are widely used in developing countries and to a lesser extent in some industrialized countries. The purpose of this research is to characterize in an animal model the behavioral and neurological toxicity of chlordecone and study the mechanisms by which chlordecone and other organochlorine chemicals produce their neurotoxic effects. Our experiments have shown in rats that chlordecone-induced tremor can be differentiated from that of p,p′-DDT and permethrin by spectral analysis techniques. Lindane is convulsive, but not tremorigenic, while o,p′-DDT and mirex produce little neurotoxicity in rats. Pharmacological and neurochemical studies have indicated that the origin of the tremor is in the brain stem. While chlordecone and p,p′-DDT are similar in that they increase the turnover of serotonin and norepinephrine, they differ in that p,p′-DDT increases dopamine turnover and increases levels of excitatory amino acid transmitters; chlordecone has no effects on these measures. Other studies have shown that dilantin markedly attenuates the tremor produced by p,p′-DDT and permethrin and exacerbates the effects of chlordecone and lindane. These data support the interpretation that p,p′-DDT and permethrin act by holding the sodium channel open and indicate that chlordecone and lindane have different mechanisms of action. Future studies will concern the possible role of calcium in the neurotoxicity produced by these agents and the neuropharmacological basis for other neurobehavioral effects produced by these agents.

PHS 6040 (Rev. 1/84) GPO 904-917

PROJECT DESCRIPTION

A. RESEARCH PROJECT:

1. Nature of the Problem: Organochlorine chemicals, such as chlordecone, p, p´-DDT and lindane and pyrethrins, such as permethrin, are widely used in developing countries and to a lesser extent in some industrialized countries. To date, there have been several attempts to identify and describe the neurobehavioral effects of the organochlorines, but there have been no systematic attempts to develop an animal model of organochlorine neurobehavioral toxicity leading to the study of their mechnisms of action.

2. Objectives: The purpose of this research is to characterize in an animal model the behavioral and neurological toxicity of chlordecone and study the mechanisms by which chlordecone and other organochlorine chemicals produce their neurotoxic effects.

3. Experimental Approach: Procedures developed in this laboratory are used to determine time- and dose-related neurobehavioral effects of representative organochlorine chamicals. In humans exposed to chlordecone and p,p´-DDT, signs and symptoms reported included tremor, hyperexcitability and alterations in ongoing performance, including possible short-term memory impairment. Tremor is measured using a spectral analysis procedure described by Gerhart et al (1982). Hyperexcitability is measured by quantification of the startle response following presentation of a novel acoustic stimulus. Potential alterations in learning and memory are measured by assessment of positively and negatively reinforced behavior (i.e., active and passive avoidance, performance in a radial arm maze).

4. Recent accomplishments and Significance:

a. Animal Model in Adult Animals.

The procedure developed by Gerhart et al (1982) has been used to quantify the effects of three organochlorine chemicals and permethrin, a pyrethroid insecticide believed to have a mechanism of action identical to that of p,p´-DDT. Previous work has shown that chlordecone-induced tremor can be differentiated from that produced by pharmacological agents such as harmine, oxotremorine, apomorphine or cold-induced shivering. Recent work has shown that the tremor produced by chlordecone can be differentiated from that of p,p´-DDT and permethrin on the basis of spectral analysis of movement; lindane was found to produce convulsions, which were not preceded by tremorigenic episodes. Mirex, an analogue of chlordecone, and o,p´-DDT were found to have little tremorigenic activity over a wide range of doses.

Subsequent experiments have shown that manipulation of the serotonergic system alters the tremor produced by chlordecone. Serotonergic antagonists (pitzotifen, methysergide) attenuate, while quipazine, a serotonergic agonist, exacerbates chlordecone-induced tremor. Depleting brain serotonin with p-chlorophenylalanine or 5,7-dihydroxytryptamine also reduces tremor produced by this agent.

These data are supported by the observation that tremorigenic doses of chlordecone produced significant increases in the utilization of serotonin in the striatum, hypothalamus, brain stem, cerebellum and spinal cord. Recent experiments have shown that pizotifen effectively attenuates the tremor produced by p,p´-DDT.

The role of the catecholaminergic system in chlordecone-induced tremor has also been studied. Rats dosed with chlordecone and then treated with phenoxybenzamine or yohimbine had significantly decreased and increased tremor, respectively. Depletion of brain norepinephrine with 6-hydroxydopamine (rats were pretreated with buproprion to protect dopamine) also reduced the tremor produced by chlordecone. Recent neurochemical studies have also shown that chlordecone increases the turnover of norepinephrine. Haloperidol, propranolol and clonidine were found to be ineffective against the tremor produced by chlordecone.

The cholinergic system has also been studied using pharmacological agents. Chlordecone-induced tremor was shown to be attenuated by trihexyphenidyl and atropine, while methylatropine was not effective. Depletion of forebrain acetylcholine using the cytotoxicant AF-64A had no effect on chlordecone-induced tremor. The role of the cholinergic system in the mediation of tremor produced by p,p´-DDT appears to be somewhat different than that of chlordecone. Recent experiments have shown that trihexyphenidyl exacerbates, rather than attenuates, the tremor produced by p,p´-DDT.

Additional studies have shown that chlordecone-induced tremor is attenuated by muscimol (a GABA agonist), chlordiazepoxide and mephenesin, a muscle relaxant that acts at the level of the spinal cord. Mephenesin has also been shown to effectively block the tremor produced by p,p´-DDT. Experimentally induced degeneration of the climbing fibers from the inferior olive to the cerebellum with 3-acetylpyridine and electrolytic lesions of the nuclei innervating the cerebellum does not attenuate chlordecone-induced tremor (in fact, they exacerbate tremor); these results suggest that the cerebellum is not necessary for the expression of this effect. Furthermore, rats exposed to chlordecone were found to be more sensitive to the effects of strychnine, which acts on postsynaptic inhibitory sites in the spinal cord.

The pharmacological data support the conclusion that a single neurotransmitter system is not involved in the mediation of chlordecone- and p,p´-DDT-induced tremor. In general, the agents that interacted most with tremor were those that act at the level of the spinal cord. Our data tend to support the possibility that the tremor may have its genesis within the brain stem.

Additional experiments have been performed to quantify the effects of chlordecone and related insecticides on behavioral hyperexcitability as measured by the acoustic startle response. Dose- and time-effect experiments have been performed and the data indicate that chlordecone, p,p´-DDT, lindane and permethrin all augment the startle response. Preliminary analysis show that each agent increases initial responsiveness (i.e., produces sensitization) and appears to attenuate or block subsequent habituation of the response to repeated presentations of the stimulus.

B. PLANS FOR THE SUBSEQUENT YEAR:

Research on the tremorigenic effects of the organochlorines and permethrin will be continued. It has been suggested that the neurobehavioral effects of the organochlorines and permethrin may be related to their effects on membrane excitability. For example, p,p´-DDT has been shown to produce repetitive firing of nerves by interfering with the closing of the sodium gate. It is possible that this effect may be manifest in the whole organism as behavioral hyperexcitability and tremor, depending upon the dose and duration of exposure. Future pharmacological experiments will employ challenges that are believed to interact at the level of the membrane. For example, 5,5-diphenylhydantoin (dilantin) is an anticonvulsant believed to interrupt repetitive discharge by acting on the sodium gating mechanism. This agent should be effective against those agents acting at this level. The effects of dilantin and related agents on tremor produced by the organochlorines and permethin will be studied. In addition, it has been reported that chlordecone may affect the intraneuronal levels of free, cytosolic calcium, producing increased membrane excitability and calcium-mediated, stimulus-coupled release of neurotransmitters. The possible role of calcium in the tremor produced by chlordecone and related agents will be studied using intracerebral injection techniques to deliver calcium ions, channel ionophores, chelators, and calcium channel blockers. Calcium should increase responsiveness of animals to agents acting by this mechanism and this effect should be enhanced by channel ionophores and blocked by channel blockers.

Future studies will also attempt to determine the possible site of action of the tremor produced by these compounds. Recent experiments conducted in another laboratory have shown that direct administration of p,p´-DDT into brain stem sites produces myoclonus; injection into regions such as the hippocampus, cerebellum and caudate nucleus did not produce these effects. We propose to microinject small quantities of chlordecone and p,p´-DDT into various regions of the brain in an attempt to elicit tremor. Pharmacological challenges effective against tremor produced by systemic administration will then be employed against tremor produced by the central route.

The effects of chlordecone and related agents on the startle response will also be studies in greater detail. First, pharmacological challenges employed in studies on tremor (i.e., dilantin, calcium) will be used to study behavioral reactivity produced by these agents. Such studies will again test the assumption that the mechanism for behavioral hyperreactivity is the same as that for tremor. In addition, a series of studies is planned which will study the role of serotonin in the enhancement of the acoustic startle response. Previous research has shown that brain stem serotonin has excitatory effects on spinal motorneurons and, when serotonin is injected directly into the spinal cord, it enhances the acoustic startle reflex. Thus, one mechanism by which chlordecone and related agents may act to enhance startle responsiveness is to increase the release of serotonin which facilitates or enhances the effects of the volley produced by the acoustic stimulus. This possibility will be tested by attempting to antagonize and exacerbate the hyperreactivity by serotonergic

antagonists and agonists respectively. The role of serotonin-containing pathways having their origin in the brain stem raphe nuclei will be studied using site specific application of 5,7-dihydroxytryptamine, a cytotoxicant for serotonin nerve terminals.

Additional studies on the behavioral mechanism of organochlorine-induced hyperexcitability are planned. Work conducted in other laboratories has shown that sensitization is mediated at some point in the motor side of the startle reflex circuit, while habituation is affected by longer sensory motor pathways. We intend to determine the effects of chlordecone on sensitization and habituation in our laboratory. One prediction is that if chlordecone is a nonspecific neural toxicant then it should affect the excitability of all components of the startle reflex. Thus, it should produce sensitization as well as attenuate habituation to repeated stimulus presentations.

Another feature of organochlorine-induced neurotoxicity is that they may activate adaptive responses in the nervous system. This has been observed in studies on tremor in which some lesions (removal of contributory pathways) or activation of some receptor populations (serotonin, alpha 2-noradrenergic) actually exacerbated the tremor produced by chlordecone. In addition, one of the common features of the neurobehavioral toxicity produced by these agents is behavioral hyperreactivity. These data suggest that one consequence of organochlorine exposure is a net alteration of processes that normally act to inhibit responding under some circumstances; loss of habituation of the startle response typifies this effect. Over the next year, we intend to initiate studies designed to evaluate the effects of chlordecone on responding under other conditions in which a behavioral inhibitory system may operate. An example of this concept is the inhibition of responding that occurs when one stimulus precedes in time the presentation of a second stimulus. The corresponding result is termed prepulse inhibition and should be markedly affected by these agents.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This research will provide information leading to the understanding of organochlorine-induced neurotoxicity.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-ES-90039-01 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Modulation of Brain Opioid Peptides by Neuroleptics and Electroconvulsive Shock

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. S. Hong	Pharmacologist	LBNT	NIEHS
Others:	T. Kanamatsu	Visiting Fellow	LBNT	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Behavioral and Neurological Toxicology

SECTION
Neuropharmacology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 - ☐ (a1) Minors
 - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously demonstrated that repeated injections of haloperidol increase the concentrations of enkephalin in the striatum and nucleus accumbens, suggesting an intimate interrelationship between enkephalinergic and dopaminergic neurons. However, measurement of enkephalin level does not reflect the dynamic change of this peptide-containing neuron. Therefore, the first objective of this project was to develop methods to measure the turnover of brain enkephalin. We tried to measure the level of mRNA coding for enkephalin precursor (preproenkephalin A) as an index for the rate of biosynthesis by cell free translation or blot hybridization using cDNA clone for preproenkephalin A. The second objective was to examine the possibility whether enkephalin and other opioid peptides can be affected by another psychiatric treatment such as electroconvulsive shock (ECS). To study the modulation of opioid peptides by haloperidol and ECS, rats received daily administration of haloperidol (1 mg/kg, i.p. for 3 weeks) or ECS (150V, 1 sec. for 6-10 days) and were killed 24 h after the last administration. Repeated injections of haloperidol caused a two-fold increase in the striatal concentration of enkephalin. This increase was accompanied by a two-fold increase in the level of mRNA coding for the precursor of enkephalin. This suggests that haloperidol accelerates the turnover of enkephalin. Furthermore, this study demonstrates that long-term treatment with haloperidol affects the gene expression of the enkephalin system. This finding raises an important consideration that gene expression may be the ultimate site of action for antipsychotic drugs. Similar to haloperidol, repeated ECS also increased the brain concentration of enkephalin and level of mRNA coding for preproenkephalin A. This finding lends further credence that gene expression may be a common site of action for various psychiatric treatments. For future studies, we plan to use the newly developed cell free translation and blot hybridization methods to study the biosynthesis of enkephalin after haloperidol or ECS in greater detail. These studies should provide further information regarding the possible role of enkephalin in mediating the actions of haloperidol and ECS.

PROJECT DESCRIPTION

RESEARCH PROJECT:

1. Problem Area

Several lines of evidence suggest that opioid peptides may act as neurotransmitter or neuromodulators in the central and peripheral nervous system. Behavioral studi suggest that enkephalins may participate in the regulation of pleasure, reward, an emotions. Enkephalins are speculated to function as modulators of neural systems that mediate the elaboration and expression of the reactive/affective components o stress. Abnormal function of this system may render the subject unable to adapt t certain environmental stressors; this in turn may result in psychosis. In additio reports concerning clinical administration of β-endorphin to mental patients and t abnormal levels of endorphin-like materials in the cerebral spinal fluid of some chronic psychotic patients are consistent with the notion that the opioid peptide systems may be associated with the etiology of mental illness.

We have been interested in studying the regulation of [Met^5]-enkephalin (ME) by ps chotropic agents which are known to affect certain neurotransmitter systems. We have demonstrated that long-term treatment with haloperidol causes a profound effe on brain ME levels. This line of study has provided evidence indicating an intima relationship between dopamine (DA) and ME in certain brain regions which are impor tant sites of action of haloperidol. It is speculated that the DA system may be t first target affected by haloperidol. Alterations in DA function may, in turn, trigger a chain of events which eventually affect the ME system and hence ultimate be responsible for mediating the actions of haloperidol.

For the last few years, further progress of this study was hampered by the lack of a method to measure the turnover of opioid peptides. Our previous studies have be limited to the measurement of steady state concentrations of the peptides by radio immunoassay (RIA). Since the steady state concentration does not precisely reflec the dynamic changes of peptide-containing neurons, development of turnover methods for opioid peptides is crucial for further understanding of the regulation of pep- tides.

2. Research Objectives

a) The first objective was to develop methods for measuring the biosynthesis of enkephalin in the brain. It is generally believed that the level of mRNA coding f enkephalin can reflect the rate of biosynthesis of this peptide. We tried to set up an in vitro cell free translation method to measure the translating activity of mRNA coding for preproenkephalin A. Additionally, we tried to develop a blot hybr dization method using cDNA clone coding for preproenkephalin A to measure the leve of mRNA coding for this peptide.

b) The second objective was to examine the possibility whether enkephalin or othe opioid peptides can be affected by another psychiatric treatment such as repeated electroconvulsive shocks (ECS) which remains the most effective treatment for de- pressive patients who are refractory to tricyclic antidepressants.

3. Experimental Approach

a) Development of cell free translation and blot hybridization for measuring the level of mRNA coding for preproenkephalin A.

The detailed procedures of these two methods are described in the Publication section.

Cell free translation	Sabol et al., 1983
Blot hybridization	Hong et al., 1984.

b) Modulation of brain enkephalinergic neurons after repeated injections with haloperidol.

Adult rats received daily injections of either saline or haloperidol (1 mg/kg, s.c.) for 3 weeks and were killed 24 h after the last injection. Striatum, hypothalamus and frontal cortex were dissected. The levels of ME were determined by RIA. The levels of mRNA coding for preproenkephalin A were measured by cell free translation and blot hybridization.

c) Modulation of brain enkephalinergic and dynorphinergic neurons after repeated ECS.

Rats received daily ECS for 6-10 consecutive days and were killed 24 h after the last shock. The brain levels of ME and dynorphin and mRNA level of preproenkephalin A were measured as described above.

4. Recent Accomplishments and Significance

a) Development of in vitro cell free translation and blot hybridization methods for measuring the level of mRNA coding for preproenkephalin A.

We succeeded in setting up these two methods which allow us to study the changes of enkephalin turnover after different treatments or manipulations. With these two methods, the future studies on the regulation of peptide can be extended from the measurement of steady state level to the estimation of dynamic changes of the peptide-containing neurons. This information is crucial for understanding the interactions between peptides and other neurotransmitters.

b) Modulation of brain enkephalinergic neurons after repeated injections with haloperidol.

Several lines of evidence, which showed that striatal levels of [Met^5]-enkephalin (ME) increase after nigro-striatal DA pathway was interrupted by hemitransection or direct administration of 6-hydroxydopamine to substantia nigra or after repeated injections of either reserpine or haloperidol, suggest that DA plays an important role in regulating the metabolism of ME containing neurons in the striatum. The increase in ME content after repeated injections of haloperidol was found in areas heavily innervated by DA neurons such as striatum or nucleus accumbens but not in hypothalamus, brain stem, and hippocampus. Further studies suggest that striatal cholinergic interneurons may partially mediate the action of haloperidol on enkephalinergic neurons. Several studies have been carried out in an attempt to determine whether the elevation of striatal ME content after haloperidol treatment was due to an increase in the synthesis or due to a decrease in the utilization of ME. The rate of decline of striatal ME content in haloperidol-treated rats was steeper than that of controls after intraventricular injection of cycloheximide. These

results suggest that haloperidol accelerates the turnover of ME. This hypothesis was confirmed by our recent findings that the activity or level of mRNA coding for preproenkephalin A, determined by cell free translation or in vitro hybridization using cDNA clones, was increased after repeated injections of haloperidol.

In summary, this study demonstrates that long-term treatment with haloperidol affects the gene expression of the enkephalin system. This raises an important consideration that gene expression may be the ultimate site of action for antipsychotic drugs.

c) Modulation of brain enkephalin and dynorphin after repeated ECS.
Repeated electro-convulsive shocks (ECS), eliciting maximal tonic extensor seizures, increase ME-LI in certain limbic areas of rat brain, such as septum, amygdala, nucleus accumbens and hypothalamus. This suggests the possible involvement of this neuropeptide in ECS-elicited phenomena, such as analgesia, retrograde amnesia, changes in seizure threshold or postictal depression. Nevertheless, alterations in ME-LI do not provide information concerning the dynamic changes of [Met^5]-enkephalin (ME)-containing neurons since an increase in peptide level could be due to either an increase in biosynthesis or a decrease in utilization. This study estimated the biosynthesis of ME by measuring the level of mRNA coding for the precursor of ME, preproenkephalin A, using both in vitro cell free translation or blot hybridization effects of repeated ECS on the concentrations of dynorphin in various brain regions. Ten daily ECS caused a two-fold increase in ME-LI and an 80% increase in the level of mRNA coding for preproenkephalin A in the hypothalamus. These observations suggest that repeated ECS increase the biosynthesis of hypothalamic ME. Ten daily ECS also increased dynorphin A (1-8)-like immunoreactivity (DN-LI) in hypothalamus (50%) caudate nucleus (30%), septum (30%) but no significant change was found in the frontal cortex or the neurointermediate lobe of the pituitary. The most prominent effect of repeated ECS on the dynorphin system was in the hippocampus. Unlike the other brain regions, a 70% decrease of DN-LI was found in the hippocampus after 10 daily ECS whereas a significant increase of ME-LI (40%) was observed. Furthermore, immunocytochemical studies revealed that an increase in ME-LI occurred in the perforant pathway and a decrease in DN-LI occurred in the mossy fiber system after 10 daily ECS. These studies suggest that alterations in enkephalin and dynorphin in the limbic system may contribute to the behavioral changes observed after repeated ECS exposure.

In summary, this study demonstrated that repeated ECS alters the biosynthesis of brain opioid peptides. This study plus the aforementioned haloperidol study strongly suggests that the gene expression may be an important site of action for some psychiatric treatments such as antipsychotics or ECS.

PLANS FOR SUBSEQUENT YEAR:

1. Studies of biosynthesis of brain opioid peptides after repeated administration of haloperidol and ECS.
After developing these two in vitro methods for the measurement of mRNA coding for preproenkephalin, we plan to study the biosynthesis of enkephalin after haloperidol or ECS in greater detail. Time course studies and detailed brain regional changes of enkephalin biosynthesis after haloperidol or ECS are planned. These studies should provide further information regarding the possible role of enkephalin in

mediating the actions of haloperidol and ECS. Since levels of dynorphin are affected by ECS, development of either cell free translation or blot hybridization method to measure the mRNA level coding for the precursor for dynorphin is planned.

2. Effect of naloxone on the metabolism of striatal neurotransmitters in haloperidol treated rats.

Although our accumulated evidence along this line of research suggests an intimate relationship between DA and ME in the basal ganglia, whether the drug-induced changes in the metabolism of peptide have any functional significance has not been studied. The proposed study is designed to answer this question.

Alterations of levels of ME in the basal ganglia after long-term treatment with haloperidol may indicate a possible change in release or synthesis of this peptide. It is suggested that ME-containing neurons may regulate the DA neuronal activity both in the striatum and in the substantia nigra. Thus, it is likely that the change in ME release may have some functional link with DA metabolism. We propose to test this possibility by injecting opiate receptor antagonist, such as naloxone, systemically or directly administered into the striatum of rats previously treated with haloperidol. Then the turnover rate of DA or other neurotransmitters (5-HT, Ach or GABA) in the striatum will be measured by HPLC and GC-MS. This study may provide some evidence suggesting that ME may participate in the actions of haloperidol.

3. Possible roles of opioid peptides in mediating ECS-elicited behavioral alterations.

Several lines of evidence suggest that endogenous opioid peptides may mediate some of ECS-elicited behavioral alterations such as post-ictal depression, changes in seizure threshold or hot plate latency, or retrograde amnesia. Our previous finding has provided neurochemical basis for this possibility. To further examine this hypothesis, we plan to study the effects of naloxone or other more specific antagonists for mu and delta subtype opiate receptors on the ECS-elicited behavioral alterations. This study may provide an opportunity to elucidate some physiological function of brain opioid peptides.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Elucidation of the regulatory mechanisms of neuropeptides may help us to learn more about the interactions between neurotransmitters and neuropeptides and the possible function of brain peptides. This type of information may provide the basis for our future studies regarding the significance of changes in neuropeptide systems after animals are exposed to environmental neurotoxicants.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-ES-90040-01 LBNT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
On the Possible Mechanism of Chlordecone-Elicited Tremor

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. S. Hong	Pharmacologist	LBNT	NIEHS
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SECTION
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INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Chlordecone (Kepone^{R}) is a chlorinated hydrocarbon insecticide. One of the most severe and consistent neurological symptoms observed in exposed workers or laboratory animals was tremor. The purpose of this project was: a) To characterize the effects of chlordecone on the brain neurotransmitter systems such as biogenic amines, acetylcholine (Ach) and amino acid transmitters by measuring the rate of turnover of these neurotransmitters and to examine the neurochemical effects of chlordecone on hypothalamo-pituitary-adrenal function by determining the plasma levels of ACTH and corticosterone. b) To evaluate the contribution of each neurotransmitter system or hypothalamo-pituitary-adrenal-axis in the tremorigenesis of chlordecone. A single injection of chlordecone caused robust increases in the turnover rate of 5HT and NE, whereas the turnover of DA, Ach and GABA remained unaltered. The steady state concentrations of amino acid transmitters were also not affected. These results suggest the possible involvement of 5HT and NE in the tremorigenesis of chlordecone. This hypothesis was further supported by the finding that BC-105 (a serotonin receptor blocker) and phenoxybenzamine (an α_1 adrenergic receptor) significantly attenuated chlordecone-elicited tremor. Chlordecone also caused a great activation of pituitary-adernal function. A robust increase in the plasma levels of ACTH and corticosterone was found 2 hr after a single injection of chlordecone (75 mg/kg; i.p.). The hypertropic effect of chlordecone on the adernal and the pituitary was further confirmed by the morphological studies both at the light and the electron microscope levels. However, the possible link between the change in pituitary-adrenal function and chlordecone-elicited behavioral alterations such as tremor or hyperexcitability remains to be studied. For future studies, we plan to examine the possibility that chlordecone, like DDT, may exert its neurochemical or behavioral effects by acting on the soduim channel of the neuronal membrane.

PROJECT DESCRIPTION

RESEARCH PROJECT:

1. Problem Area

Chlordecone (Kepone^{R}) is a chlorinated hydrocarbon insecticide similar in structure to other organochlorine insecticides, such as mirex. One of the most severe and consistent neurological symptoms observed in exposed workers during the production of chlordecone at Hopewell, Virginia, was tremor. In moderately affected workers, tremor was irregular and non-purposeful. Amplitude of the tremor was accentuated by intentional acts and sustained postural movement. In the more severely affected workers, tremor occurred at rest. This kind of tremor has been demonstrated in laboratory animals such as rats and mice.

Neurotransmitter systems have been suggested to play some role in the expression of tremor elicited by various tremorigens. For example, the cholinergic system in the basal ganglia may mediate the tremor produced by oxotremorine and tremorine. Serotonin may participate in the tremoring action of harmine. Hrdina et al. suggested that cholinergic and serotonergic mechanisms may be involved in DDT-induced tremor. Thus, it is likely that brain neurotransmission may be closely associated with chlordecone-induced tremor. However, very few neurochemical effects of this insecticide have been reported. Previous work in our laboratory has indicated that chlordecone affects the disposition of dopaminergic and cholinergic receptor binding sites in rats. Recent research by Fujimori et al. (1982) also suggests that chlordecone affects the metabolism of biogenic amines (particularly dopamine) in mice. These results suggest a possible involvement of neurotransmitter systems in the expression or modulation of tremor elicited by chlordecone. Nevertheless, the effects of chlordecone on brain neurotransmitter systems and the possible contribution of each neurotransmitter in the tremorigenesis elicited by this insecticide have not been systemically studied.

In addition to the neurotransmitter systems, previous work in this laboratory indicates that alterations in hypothalamo-pituitary-adrenal function (HPA) may be responsible for some of the long-term behavioral changes that follow developmental exposure to chlordecone. However, the neurochemical effects of chlordecone on the HPA axis have not been characterized.

2. Research Objectives

a. To characterize the effects of chlordecone administration on the brain neurotransmitter systems, such as biogenic amines (DA, NE, and 5-HT), Ach and amino acid transmitters, by measuring the rate of turnover of these neurotransmitters.

b. To evaluate the contribution of each neurotransmitter system in the tremorigenesis of chlordecone.

c. To characterize the neurochemical effects of chlordecone on the HPA axis and evaluate the contribution of the HPA axis in the tremorigenesis of chlordecone.

3. Experimental Design

To assess the effects of chlordecone on brain neurotransmitter systems, rats received a single injection of tremorigenic dose of chlordecone (80 mg/kg, i.p.). Tremor activity was monitored at different time periods post-dose with spectral analysis as described by Gerhart, et al., (1982). After completion of the measurement of tremor activity, rats were killed either by decapitation or by microwave irradiation and brains were dissected according to the method of Glowinski and Iverson (1966). Turnover studies of DA, NE, and 5-HT were conducted by measuring either the metabolite levels of each biogenic amine or by determining the rates of decline of amine levels following the blockade of synthesis. Levels of amines and their acid metabolites were measured by either high performance liquid chromatography (HPLC) (Wilson, et al., 1983), or gas chromatography-mass spectrometry (GCMS) (Koroum, et al., 1975). Turnover rates of Ach and GABA were measured by GCMS as described by Racagni, et al. (1976). Levels of amino acid transmitters were measured by HPLC.

To evaluate the contribution of each neurotransmitter system in the tremorigenesis of chlordecone, pharmacological challenge studies were performed. Rats received a single injection of chlordecone (80 mg/kg, ip). Five hr post-dose, various compounds (agonists or antagonists specific to a neurotransmitter receptor) were injected and tremor activities were measured 30 min afterward. Modifications of chlordecone-induced tremor activities by these pharmacological agents may suggest a possible involvement of a neurotransmitter system in the tremorigenesis of this insecticide.

To characterize the neurochemical effects of chlordecone on the HPA axis, levels of ACTH in the pituitary and plasma and levels of corticosterone in the adrenal and plasma were measured by RIA after chlordecone treatment. Morphological studies of the adrenal and pituitary glands were also performed at the light and electromicroscope levels.

4. Accomplishments

a. Analytical methods development

For the purpose of measurement of biogenic amines and their acid metabolites, we have developed an automated HPLC method using electrochemical detection which allows simultaneous determination of NE, DA, DOPAC (dihydroxyphenyl acetic acid), HVA (homovanillic acid), 5-HT and 5-HIAA (5-hydroxyindoleacetic acid) (Wilson, et al., 1983). An automated HPLC method employing a post-column derivatization technique was developed for the simultaneous measurement of amino acid transmitters, such as taurine, aspartate, glutamate, glutamine, glycine, and GABA.

A GC-MS method was set up for measuring the tissue concentrations of 3-methoxy-4-hydroxyphenylglycol (MHPG), a major metabolite of NE.

b. Effects of chlordecone on brain neurotransmitter systems

The effects of a single injection of chlordecone (80 mg/kg, I. P., killed 24 hr later) on the brain neurotransmitter systems are summarized in Table 1. Among various types

of neurotransmitters studied, chlordecone administration increased the rates of turnover of 5-HT and NE, whereas the turnover of DA, Ach, or GABA and steady state concentrations of amino acid neurotransmitters were not affected. The increases in the levels of 5-HIAA occurs in every brain region examined and the increase in MHPG-SO_4 was also found in four brain regions determined. Finally, some of the neuropeptides such as enkephalin or substance P which are suggested to be involved in the regulation of motor activity were not affected by this treatment. Results from this study illustrate certain degree of selectivity of chlordecone's effects on brain neurotransmitters and also provide the basis for further studies concerning the contributions of 5-HT and NE in chlordecone-induced tremor.

c. Roles of 5-HT, Ach and NE in the tremorigenesis of chlordecone

i) 5-HT:

It has been suggested that brain 5-HT system may play some roles in tremorigenesis (Jacobs, 1976). Tremor was observed in rats administered with compounds which either dramatically increase synaptic 5-HT (e.g., monoamine oxidase inhibitors, 5-HT precursors, or 5-HT releasing agent) or directly stimulate postsynaptic 5-HT receptors. The following observations are consistent with the hypothesis that 5-HT may be involved in chlordecone-induced tremor.

(1) A single injection of chlordecone increased brain levels of 5-HIAA. Turnover study by measuring the rate of decline in 5-HIAA after inhibition of MAO activity by pargylline indicated that the increase in 5-HIAA level after chlordecone exposure represents an increase in the turnover rate of 5-HT. These results suggest that chlordecone treatment produces an overactivity of 5-HT neurons (Hong, et al., 1983).

(2) There was a close time- and dose-related correlation between the increase in 5-HIAA levels and tremor activities after chlordecone exposure (Hong, et al., 1983).

(3) Chlordecone-elicited tremor could be modified by compounds which affect 5-HT transmission. For example, BC-105 (a 5-HT receptor blocker) attenuated whereas pizotifen (an agonist for 5-HT receptor) accentuated the tremor activity (Gerhart, et at. 1982).

ii) Ach:

A cholinergic mechanism is suggested to mediate the oxotremorine-elicited tremor (Cox and Potkonjak, 1970). Ach may also be involved in the tremorigenesis after DDT exposure (Hrdina, et al.,1975). The possible involvement of the cholinergic system in chlordecone-elicited tremor was suggested by the finding that Artane, a cholinergic blocking agent, was able to partially antagonize the effect of chlordecone. However, no significant change of Ach turnover was found in different brain regions after chlordecone treatment. This suggests that the involvement of the cholinergic system in chlordecone-elicited tremor may be an indirect effect, possibly due to an imbalance between Ach and GABA (see Gandolfi et al., 1984).

iii) NE:

The possible involvement of NE in chlordecone-elicited tremor was suggested by the finding that phenoxybezamine, an α_1 receptor antagonist, was able to partially antagonize the effect of this insecticide. Measurement of brain concentrations of

MHPG indicated that chlordecone greatly increases the turnover of NE in the hypothalamus, brain stem, cerebellum and striatum. The increase in MHPG level in the brain stem and hypothalamus preceded the onset of tremor after chlordecone exposure. These results strongly suggest that the NE system may be closely related to the tremorigenesis of chlordecone.

d. Neurochemical effects of chlordecone on the HPA axis:

A single injection of chlordecone (75 mg/kg, i/p.) increased the adrenal and serum levels of corticosterone (5-10 fold above control level) 24 h post dose. The increase in corticosterone levels persisted up to 7 days post dose. Such hyperfunction of the adrenal cortex is consistent with morphological observations which indicated that adrenal cortical cells hypertrophy after chlordecone treatment. In an attempt to determine whether the action of chlordecone on the adrenal is a direct effect or is mediated through the pituitary, serum levels of ACTH were measured. There was a robust increase in serum levels of ACTH which were in close parallel with the increase in serum levels of corticosterone. These results suggest that the hypertropic effect of chlordecone on the adernal may be mediated through the pituitary. However, more studies are required before a definite answer can be offered. (see the Research Plan section).

RESEARCH DEVELOPMENT AND PLANNING FOR THE FUTURE:

1. Comparison of effects of chlordecone, DDT, and mirex on the neurotransmitter systems.

The neurochemical effects of chlordecone and other organochlorine compounds on neurotransmitters will be compared. As mentioned in previous sections, chlordecone and DDT produce very similar behavioral profiles such as tremor, hyperexcitability, etc. Therefore, it would be interesting to compare the commonality of these two insecticides on the neurotransmitter systems. Another compound of interest is mirex. Mirex is an analogue of chlordecone with very small difference in their chemical structures. However, these two compounds have qualitatively different behavioral profiles. For example, mirex is not tremorigenic in rodents. Thus, it may offer a unique opportunity to dissociate the neurochemical changes with tremor activity if mirex produces alterations of some neurotransmitter systems.

2. On the molecular mechanism underlying the membrane effects of chlordecone, DDT and mirex.

Another biochemical approach for the comparison of these three chlorinated insecticides will be focused at the cellular membrane level. Wu et al., (1980) reported that DDT causes repetitive firing in lobster axon presumably by holding the sodium channel open. It is possible that the DDT-induced increase in neuronal firing may be related to increases in turnover of 5-HT or NE (Hrdina et al., 1975). We plan to study the biochemical effect of chlordecone, DDT, and mirex on the sodium channel preparation from rat brain. In collaboration with Dr. Robert Aronstam at the University of Georgia, we will investigate the effect of these three compounds on the sodium channel by evaluating their potency to compete for the binding of ^{3}H-batrachotoxin (a specific ligand for sodium channel protein) to sodium channel. If this

in vitro binding study gives positive results, then a sodium flux study will be conducted to verify the possibility that the sodium channel may be the site of action for these organochlorine insecticides.

3. Further charactization of neurochemical effects of chlordecone on HPA function.

Our preliminary results indicated that chlordecone exerts a robust effect on the HPA axis. However, it is not clear whether the change in HPA function is related to the behavioral alterations such as tremor or hyperexcitability after chlordecone treatment. A series of experiments, such as examining the effects of hypophysectomy or adrenalectomy, or using pharmacological challenges on the chlordecone-elicited tremor or hyperexcitability is planned in an effort to answer this question.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE

Elucidation of the neurochemical mechanism underlying chlordecone-elicited tremor may help us to learn more about the central regulation of tremor. This type of information may also provide the basis for clinical treatment of patients exposed to this insecticide.

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TABLE 1

EFFECTS OF CHLORDECONE ADMINISTRATION (80 mg/kg IN DMSO; I.P.)
ON THE BRAIN NEUROTRANSMITTER AND NEUROPEPTIDE SYSTEMS

SUBSTANCE MEASURED	STRIATUM	FRONTAL CORTEX	HYPOTHALAMUS	HIPPOCAMPUS	BRAIN STEM	CEREBELLUM
DA	——	——	——	——	ND	ND
DOPAC	——	——	——	ND	ND	ND
5-HT	——	——	——	——	——	——
5-HIAA	↑	↑	↑	↑	↑	↑
NE	ND	ND	↓	ND	——	ND
MHPG-SO_4	↑	ND	↑	ND	↑	↑
ACETYLCHOLINE TURNOVER	ND	——	ND	——	ND	ND
GABA	——	ND	——	——	——	——
TAURINE	——	ND	——	——	——	——
GLYCINE	——	ND	——	——	——	——
GLUTAMATE	——	ND	——	——	——	——
ENKEPHALIN	——	——	——	——	——	ND
SUBSTANCE P	——	——	——	——	——	ND

——: NO CHANGE ↑: INCREASE ↓: DECREASE ND: NOT DETERMINED

NEW YORK UNIVERSITY MEDICAL CENTER - New York, NY 10016
(NIH-NO1-ES-2-5017)

TITLE: Use of Homecage Behaviors to Screen for Potential Toxicity of Chemicals

CONTRACTOR'S PROJECT DIRECTOR: Hugh L. Evans, PH.D.

PROJECT OFFICER (NIEHS): Hugh A. Tilson, Ph.D., Head
Neurobehavioral Workgroup, LBNT

DATE CONTRACT INITIATED: May 25, 1982

CURRENT ANNUAL LEVEL: $113, 294

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this research are: 1) to obtain information about the utility of continuous home-cage monitoring of domiciliary behavior as a reliable index of neurobehavioral toxicity, 2) to determine the effects of representative neurotoxicants on these behaviors, and 3) to attempt to correlate alterations in tissue levels with the presence and magnitude of behavioral effects.

METHODS EMPLOYED:

A homecage monitoring system is used to measure continuously food and water ingestion and spontaneous motor activity. Rats are dosed chronically with toxic agents and alterations in the frequency and cyclicity of these behaviors are determined using computer-assisted programs. At the end of the dosing period, animals are sacrificed to obtain blood and brain levels of the compound.

MAJOR FINDINGS AND PROPOSED COURSE:

A system to monitor up to 40 rats has been built and is being used. Validation studies have been completed to assess the utility and sensitivity of the system and experimental design. Growth curves for rats maintained with the system are identical to those maintained with conventional watering and feeding. The rat's diurnal cycle in activity and ingestive behavior has been demonstrated to shift in response to changes in the daily light-dark cycle. Food deprivation and post-deprivation rehabilitation produced the expected changes in activity and consummatory behavior.

Acute dose-response determinations for scopolamine and amphetamine indicated sensitivity of the system to short acting psychoactive agents. A time-effect study of acute oral exposure to trimethyltin is now underway.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

One of the earlier signs of toxicity is frequently a change in body weight. The system being developed and validated will permit the quantification of changes in ingestive behaviors and motor activity preceding body weight alterations. The use of behavioral indices of toxicity may prove to be an early warning for the detection of toxicity, as well as providing clues to understand how toxicants interfere with neurobiological processes controlling food and water intake.

NORTH CAROLINA STATE UNIVERSITY - Raleigh, NC
(NIH-NO1-ES-2-5019)

TITLE: Research Support to Investigate Teratogenic Effects of Environmental Agents Using Avian Species

CONTRACTOR'S PROJECT DIRECTOR: Robert E. Cook, Ph.D.

PROJECT OFFICER (NIEHS): Donald I. McRee, Ph.D., Research Physicist
Laboratory of Behavioral and Neurological Toxicology

DATE CONTRACT INITIATED: August 1, 1982

CURRENT ANNUAL LEVEL: $149,434

PROJECT DESCRIPTION

OBJECTIVES: The objectives of the contract are to provide housing and maintenance services for care of research animals (Japanese quail) and to provide support staff necessary to conduct biological tests on birds which have been exposed to environmental agents.

METHODS EMPLOYED:

The contract will provide a special breeding colony for adequate supply of fertilized Japanese quail eggs, brooding facilities for housing and care of baby quail, and housing and maintenance of experimental Japanese quail throughout duration of experiments. The contractor will also provide support personnel, materials, supplies, facilities and equipment for conduct of biological experiments. The support personnel will include a full-time professional level researcher, two full-time technical support persons, and two part-time caretaking and maintenance support persons. Research protocols will be developed by the staff of the Laboratory of Behavioral and Neurological Toxicology (LBNT) of NIEHS with the participation of the professionals provided by the contractor. The research projects will then be conducted by the personnel provided by the contractor with consultation and assistance of the staff at NIEHS.

MAJOR FINDINGS AND PROPOSED COURSE:

Eleven research protocols have been written by the staff of NIEHS and N.C. State University, and the research have either been completed or is in progress. Four of the protocols are concerned with the biological effects of microwave radiation and the other seven protocols are studies to determine the effects of chemical agents. The four microwave bioeffect studies are designed to determine the response of quail to various stressful conditions after microwave exposure during embryonic development as the quail mature. In all cases the exposures were at a microwave frequency of 2.45 GHz, at an incident power density of 5 mW/cm^2 (specific absorption rate=4.03 mW/g) and during the first 12 days of development. The hemetological response of immunized quail was measured in juvenile (5 weeks old) and adult (22 weeks old) quail at 3, 6, and 9 days post-immunization. Only the exposed juvenile female quail elicited a response different from controls. Total reticulocytes were initially depressed and at 9 days post-immunization they expressed an increased number of reticulocytes in comparison to controls. The response of quail exposed to microwave radiation in the above manner to hemorrhagic stress was investigated. The quail were mechanically hemorrhaged to 70%

of their calculated blood volume. Circulating erythrocyte and reticulocyte numbers were depressed below controls by 24 hr post-hemmorrhage in exposed juvenile males. However, lymphocyte numbers elevated in exposed juvenile males at 72 hr. These data suggest that hematopoiesis is affected by embryonic irradiation and that the effect persists for a long time after hatching (exposure).

Japanese quail eggs were injected with DES (0.9-1000ug) dissolved in 50 ul of corn oil on day 1 of incubation. Higher doses of DES (250-1000ug) reduced hatchability to 37-33% compared to 61% for corn oil injected controls. Lower doses of DES (0.9-125ug) had no effects on hatchability. In a second study, eggs were injected with 0.9 or 1.9 ug of DES and the survivors were assessed up to 12 wks posthatch. DES did not affect hatchability, but did increase mortality during the first 4 wks posthatch. Females were affected more than males. At 10 days of age, open field activity of some birds was examined. The acquisition and reversal of a visual discrimination task was studied at 6 wks of age. Exposure to DES blocked egg production in these birds. The oviduct weights of 12 wk old females were decreased by 50%, but ovarian weights were not affected. Testicular weights were not affected. In a third study reproductive behaviors and social-dominance behaviors of males were markedly attenuated in birds exposed to 0.48 or 1.9ug DES in ovo.

Two primary projects are planned during the coming year. The first experiment will study the effects of 2.45-GHz microwave radiation during embryogeny on reproductive function of Japanese quail. Past research has indicated that the fertilizing capacity of the male was decreased with this type of exposure. Offspring will also be mated to determine if genetic changes are carried forward to the next generation. The second project will study the influence of chlordecone (Kepone) on Japanese quail embryonic mortality and five week survivability. Eggs will be injected on day 0 of development with doses ranging from 0.001 mg to 10 mg of Kepone in corn oil. The percent of eggs hatching in each treatment group will be recorded. Eggs that do not hatch will be examined and classified according to mortality. Post hatch deaths will be recorded. Weekly body weights, up to 35 days of age, will be taken on 10 birds per group.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The results of this research will increase our knowledge of the biological effects of microwave radiation and chemical agents. The use of the avian embryo as a test system provides the capability of studying the direct effects of environmental agents on development without maternal complications.

LABORATORY OF GENETICS

LABORATORY OF GENETICS
Summary Statement

Organizational changes that combined two sections in the Laboratory of Genetics have taken place during the past year. The Laboratory now consists of only two sections, Eukaryotic Gene Structure and Mutagenesis.

Eukaryotic Gene Structure: Studies in this section are designed to advance the understanding of gene organization and function in individuals and populations of eukaryotic organisms. A central problem is determining the mechanisms for the regulation of genes in developmentally orchestrated arrays. The major approaches to these problems are to clone specific genes that play important roles in developmental pathways, characterize them at the molecular level, and compare the normal alleles with mutant forms that modify various aspects of the regulation of the locus.

A gene that has been intensively studied in Drosophila melanogaster is the white locus. There are a number of mutant alleles that upset the regulation of the gene in various ways. One of the series studied by the Judd group produces mosaic patches of normal and mutant tissue in the eyes, suggesting that the structure of the portion of the gene encoding the mRNA is normal but that some aspect of the regulation is modified. The mutant w^{zm} and some of its derivatives cause clonal, autonomous expression of the gene in some cells of the eye anlage during development while other cells have reduced or no gene expression. This mutation is associated with the insertion of a transposable element into an intron of the gene near its 5' end. The insertion has been cloned into recombinant DNA molecules and partially characterized by restriction endonuclease digestion. Some of the mutants derived from the w^{zm} strain show transposition of the white locus to another chromosome. In the new position the gene becomes non-clonal and non-autonomous in expression. These new gene forms are being cloned to determine their structure and to discover how the normal timing and tissue specific action of this locus is regulated. Other white mutant alleles giving mosaic phenotypes show interactions with their allele in the homologous chromosome. This interaction is upset when somatic pairing of the two chromosomes is upset. This phenomenon, known as transvection, is being studied as a means of determining what the regulatory signals within the locus are that can be transmitted between homologous chromosomes to influence gene function.

Another gene in Drosophila that is developmentally regulated to function in very specific tissues is the cut locus. Mutant alleles constitute at least three complementation groups, with those in each group having rather profound effects on separate unique parts of the fly such as legs, wings, eye and antennae. Dr. Jack's group has cloned this locus and characterized a number of spontaneous mutations that are associated with insertions of transposable DNA sequences into the locus or the deletion of portions of the locus. The gene extends over about 150kb of DNA making it one of the largest known in Drosophila. Mutation sites affecting various body parts are clustered in specific sections of the gene. The characterization of these gene segments and the mutations in them should give important information about how the developmental steps in the formation of specific organs and tissues are controlled.

That transposable DNA sequences play an important role in the spontaneous mutation process in Drosophila is now well documented. Furthermore, it is clear that some of the families of transposons have structures similar to the retroviruses found in mammals, including humans. An example of this important class is the basis for the system of mutable genes studied by Dr. Lim, University of Wisconsin, Eau Claire. The transposon gypsy is retrovirus-like and is highly mobile in a stock constructed by Dr. Lim. The high mobility is correlated with extremely high mutation rates at specific loci. During the analysis of some of the mutations at the cut locus generated in this system, it became evident that occasionally the cut allele transmitted through the germ line of a single individual is different than that expressed in the somatic tissues of that individual. Models for explaining the cryptic transmission are presently being tested by this group. Preliminary findings implicate the transposon in the unusual transmission process and suggest that the transmission may be by means of an extrachromosomal element, possibly similar to a bacterial plasmid.

Transposable elements are involved not only in the mutation and transmission of genes but also in several aspects of gene expression. A very interesting aspect is brought into focus through the works of two other groups, Dr. Voelker's and Dr. Searles'. Dr. Searles has cloned the vermilion locus in Drosophila and is studying the structure and expression of the gene. Some of the mutants at the locus are due to the insertion of transposon sequences into the gene. Some of the mutations of this type are suppressible by the mutations at the suppressor of sable locus, su(s). Dr. Voelker's group has in turn cloned the suppressor of sable locus and determined that all of the spontaneous mutations contain insertions of non-su(s) DNA. The objectives are to characterize the gene in molecular detail and obtain the protein product by placing the cloned gene in an expression vector system. The gene product can then be used to investigate the mechanism of action of the suppressor and to learn more about how the host organism reacts to the mutational changes due to particular transposons.

Drs. Voelker and Searles are also collaborating with Dr. Greenleaf at Duke University on the study of genes encoding RNA polymerase II. Characterization of the gene encoding the 215,000 dalton subunit of the complex is well advanced. A number of P element induced mutations and revertants of such mutations have been analyzed. Though the objective is to understand the genetic control of the biosynthesis of this important enzyme complex, the search for mutations in genes encoding other subunits of the complex has not yet been fruitful.

Dr. Boswell's group is concerned with the genetic and developmental analysis of genes that play roles in the determination and differentiation of germ cells in Drosophila. Boswell's approach is to identify mutants that impose a maternal effect on germ cell development. The genetic screen for such mutants is designed to find individual mutant females whose offspring are sterile due to the failure of germ cell development. Most of the analysis is directed toward the gene tudor. Females homozygous for this mutation are grandchildless because they produce embryos that lack pole cells, the precursors of the adult germ cells. Several alleles have been characterized genetically and developmentally. Deletion mapping has located the locus in the right arm of

chromosome 2 and a cloned segment of DNA that is located close to the tudor position has been obtained. Molecular techniques for cloning adjacent sections of DNA are being used to move along the chromosome toward the tudor locus. Objectives are to clone the locus and determine how it is involved in the determination and differentiation of the germ cells. This information should help in understanding how determination is established and maintained during development.

Two groups, those of Dr. Langley and Dr. Aquadro, have been investigating patterns and levels of naturally occurring genetic variability in populations of Drosophila melanogaster. They have focused on the regions in and around two loci, one encoding the enzyme alcohol dehydrogenase (Adh) and the other encoding dopa decarboxylase (Ddc). Rather different pictures about the amount, location and apparent effects of DNA sequence variation have emerged from these two studies. Considerable sequence variation is found in and around both genes, much of it due to insertions or deletions associated with the actions of transposable elements. Examination of the Ddc gene and an 80 kb region flanking it gave no clear pattern between sequence variants and Ddc expression, unlike the Adh gene region where there is a strong suggestion that sequence variants have modified Adh activities. Also there seems to be preferential insertion of transposable elements into DNA sequences that appear to be important in the regulation of Adh. A curious discovery in the Ddc region shows numerous insertions/deletions are present in a 30-40 kb region where a dense cluster of eight lethal-mutable genes are located while almost no variation was found in a 40 kb region where only one gene is known.

A rather unusual mutator element has been investigated by the Langley group following up on the genetic analysis of a series of eye morphology mutants in D. ananassae by C. W. Hinton. Langley has isolated an element in cloned recombinant DNA that appears to insert preferentially at sites of genes involved with determining eye morphology. The characterization of the element and the sequences where it inserts should provide important information about transposable genetic elements and about the sequences involved in the coordinate control of genes in developmental pathways.

Dr. Skow's group has concentrated most of its research effort on the study of mutants affecting the mouse eye. Using cDNA probes for α-, β- and γ-crystallins they have been searching for variants of genes encoding these lens proteins. Genetic polymorphisms have been found in regions flanking the α-crystallin structural gene and these markers have been used to map the gene to chromosome 17, tightly linked to the Acry-1 and H-2K loci. Similarly, restriction site polymorphisms are found in the γ-crystallin sequences and mapping experiments indicate no recombination with a γ-crystallin electrophoretic variant LEN-1 mapped to chromosome 1. The tight linkage of this gene to Elo which produces anopthalmia in mice suggests an interesting set of experiments about the relationships between the two loci relative to the development of the eye.

Dr. Malling's group has focused on the detection of mutations in mammals. Of particular interest is the development of systems that can detect mutations in single mammalian cells. Attempts to detect mutations in mouse sperm based on antigenic differences between mouse and rat lactate dehydrogenase (LDH-C)

proved unsuccessful. The interspecific differences apparently are not appropriate markers for single mutational events. Antibodies to malic enzyme have been obtained and techniques for staining sperm and somatic cells are being tested to determine whether detection of mutant forms of this enzyme is feasible in single cells. Other systems that are being investigated for the detection of genetic damage are the use of mitochondrial DNA or viral DNA transformed into mammalian chromosomes. The approach here is to use endonuclease restriction sites as the markers for detecting damage to the mitochondrial or viral DNAs.

Dr. Johnson's group is studying spontaneous and induced mutations in mice. Their goal is to obtain information about the specific molecular events involved in mutational changes and to determine the developmental effects of specific gene modifications. The approach is through the study of electrophoretic variants of a select set of about 30 proteins. These methods have identified a number of spontaneous and induced mutations, some of which have served as models for understanding particular diseases in humans. For example, a mutant in the mouse that produces a β-thalassemia phenotype was identified in this study. Its molecular characterization was done by Dr. Skow's group in conjunction with Dr. Johnson and his group.

Dr. Li's group is studying several genes and gene products in mammals. Primarily the work focuses on genes encoding the lactate dehydrogenase (LDH) isozymes. Clones of LDH-A cDNAs from mouse and human have been isolated and sequenced and several genomic clones from mouse and humans have been recovered and partially characterized. Recently, work in other laboratories has shown that the LDH proteins are capable of binding single stranded DNA. Since the entire amino acid sequence for several of the LDH isozymes has been determined, the domain responsible for this binding can now be examined with a view to understanding its possible role in the function and regulation of these genes. The organization of various members of the LDH gene family in several species is being determined at the molecular level. Initial results show interesting evolutionary and mutational relationships in conserved and nonconserved regions and between gene and pseudogene sequences.

Mutagenesis: The Mutagenesis Section devotes its efforts to understanding mechanisms of spontaneous and induced mutations. It is comprised of similar numbers of geneticists and biochemists who study model systems ranging from bacteriophage T4 through the bacterium Escherichia coli, the yeast Saccharomyces cerevisiae and the fruitfly Drosophila melanogaster, to cultured mammalian somatic cells. In addition to the analysis of mutation itself, considerable effort is directed towards the underlying processes of DNA replication and repair.

The Drake group is working on the process of error-prone repair (EPR) in phage T4. EPR is the dominant mode of induced mutagenesis in most organisms, very probably including mammals. Mutations that affect two genes required for EPR in T4; have recently been generated and characterized among these, the most interesting are temperature-sensitive mutations that affect survival and mutagenesis differentially. Conditional TS mutations will be sought in a third gene of this system, and in any other gene that may be discovered to be required for EPR in T4. The proteins corresponding to the wild-type and TS

alleles will be isolated and examined to determine which traits appear to be involved in survial versus lethality. Tests are underway to determine if induced mutagenesis and induced recombination are correlated, since a major hypothesis of EPR stipulates that mutagenic DNA synthesis occurs in the vicinity of damages in recombinational intermediates rather than in ordinary replication forks.

The Glickman group has been conducting analyses of mutagen specificity in diverse systems, namely the lacI gene of E. coli and in cultured mammalian somatic cells. In the lacI system, the effects of a number of repair defects and a number of mutagens have been investigated. In a mutant unable to excise uracil from DNA, a substantial increase in G:C → A:T transitions occurs, as expected from cytosine deamination; but these appear to arise preferentially in A:T-rich regions, suggesting an important role for DNA "breathing" in deamination mutagenesis. A mutant deficient in EPR showed no increase in mutation rate, either for the gene as a whole or in the distribution of mutations within the gene, indicating little role for EPR in spontaneous mutation (in contrast to its very important role in induced mutation). In a wild-type strain, a number of spontaneous deletion mutations were collected and sequenced; the majority appeared to arise by the excision of palindromes, in support of a major model of deletion mutagenesis. In strains defective in any of four genes required for postreplication mismatch repair, quite different distributions of mutations were observed, suggesting considerable specificity in the mutation-avoidance potential of the individual components of the system. Two types of pyrimidine-pyrimidine dimers are now known to be efficiently induced by ultraviolet irradiation, and evidence was accumulated suggesting that either kind of dimer can act as a premutagenic lesion, although with very different distributions within a gene.

The Glickman group's work with cultured cells was only recently initiated, but substantial progress has been made in the construction of strains andd vectors permitting mutations arising in mammalian cells to be transferred into bacteria for further analysis, including DNA sequencing.

The Kunkel group has concentrated on the analysis of mutagenesis occurring during the in vitro synthesis of DNA but detected by phenotypic alterations in vivo and charcterized by DNA sequencing. The three major mammalian species of DNA polymerase, characterized for their mutagenic propensities in a forward-mutation system, show three distinct rates and specificities of mutation. A major component of these differences is seen in a strong correlation between the fidelity and the processivity of the polymerase; this discovery marks the first insight into the nature of intrinsic polymerase accuracy, as distinct from accuracy associated with the action of the 3'-exonucleases that occur in prokaryotic polymerases but are absent from these mammalian polymerases. In a second series of studies, specific kinds of DNA damage are being examined for their mutagenic consequences. Abasic sites, such as those arising from spontaneous and/or induced depurination, have been shown in the forward-mutation assay to generate base-pair substitutions, double mutants, gene-conversion-like mutational events and, at a lower rate, frameshift mutations. The most common mutations are single base-pair substitutions. Most arise opposite purines (especially guanine), and most involve the insertion of an adenine residue opposite an apurinic site, thus generating transversions.

The Ripley group has concentrated on the analysis of frameshift mutagenesis occurring in vivo in phage T4, followed by DNA sequence analyses of the mutations. The wild-type and several mutant versions of the T4 DNA polymerase cause distinctly different rates of frameshift mutagenesis in different sequences and involving different changes. It has thus become possible to delineate a number of distinct mechanisms of frameshift mutagenesis, many more than known just a year or two ago. The classical type involves the addition or deletion of a single residue from a simple repeating sequence, or the addition or deletion of a single block of resides from a repeating block. In addition, however, single-base duplications are common outside of repeating sequences. At least three types of complex mutations have been detected; these involve multiple frameshift and base-pair substitution changes arising in a single event. Some are due to the recently discovered quasipalindrome correction mechanism. Others appear to be caused by a kind of gene-conversion event acting over a considerable distance. A third group consists of frameshift mutations separated, by two or three bases, from a transversion; but the mechanism that generates this class is still poorly understood. While the above classes of frameshift mutagenesis operate spontaneously, it has recently been found that proflavin-induced frameshift mutations tend to fall within particular nucleotide octamers that share a strongly conserved sequence. Again, the mutagenic propensity of these sequences is not yet understood.

The Schaaper group, originally a part of the Glickman group, is in the process of establishing its independence. In addition to extensive participation in the projects outlined as components of the Glickman program, Schaaper has been working on EPR-like mutagenesis in vitro. This is a problem that has eluded resolution in many excellent laboratories around the world over the past decade. To date, procedures have been developed to detect mutations induced by cell extracts acting upon a target gene in vitro, followed by in vivo expression and, finally, characterization by DNA sequencing.

The Volkert group has concentrated on two aspects of DNA repair and mutagenesis in E. coli: the recF gene, and genes involved in the response to alkylation damage. Mutations in the recF gene affect both mutagenesis and recombination, and a detailed analysis of the properties of recF mutations and their extracistronic suppressors supports a model that assigns to the recF protein a role in modulating two functions of the recA protein: its conversion following UV irradiation of the cell to a protease, and its control over the recF mode of recombination. (The srfA suppresor of a recF mutation separates these two functions.) Genes involved in the response to alkylation damage are being detected by means of insertion mutations, wherein the insertion carries a portion of a lac gene whose activity can be monitored; mutants are then selected in which the lac gene is activated specifically by alkylation damage, indicating that the recipient gene is induced by such damage. To date, at least five such genes have been discovered, two being known previously in other contexts. The patterns of response of these genes are complex, and are now being sorted out.

A number of personnel changes are occurring in FY-84. The Glickman and Ripley groups are leaving the NIEHS. A major portion of the Glickman group, namely the Schaaper group, is advancing to independent status within the Mutagenesis Section. Finally, the Sugino group, which left the NIEHS two years ago, is returning.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60098-05 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mammalian Lactate Dehydrogenase Isozyme Analyses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: Steven S.-L. Li Research Geneticist LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 61032-01 LG entitled "Structure-function of mammalian lactate dehydrogenase isozymes".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60099-05 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization-regulation of mammalian lactate dehydrogenase genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Steven S.-L. Li	Research Geneticist	LG, NIEHS
Others:	Hiroshi Tsujibo	Visiting Fellow	LG, NIEHS
	Kiyohito Yagi	Visiting Fellow	LG, NIEHS
	Kayoko Fukasawa	Visiting Fellow	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

LDH-A cDNA clones from mouse and human have been isolated and sequenced. Mouse pMLA 73 was found to contain the 393bp of coding region and 496 bp 3' untranslated sequence including poly (A) tail. Four human cDNA clones consist of sequences of approximately 1,700bp, that is, the complete coding sequence (999bp), the 5' (97bp) and 3' (565bp) untranslated regions. Several LDH genomic clones have also been isolated and partially characterized from mouse and human DNA libraries. Mouse genomic clone M15 appears to contain a functional gene of at least 8Kb consisting of several exons and introns which have been sequenced. The nucleotide sequence of a human LDH-A pseudogene has been determined. 12.9% nucleotide differences were found between the pseudogene and LDH-A cDNA, and their significant implications on mammalian mutations and molecular evolution are observed. It is of interest to elucidate the genetic mechanism(s) underlying the tissue-specific expression of three LDH genes in human and other vertebrates.

PROJECT DESCRIPTION

PROBLEM: Structural organization, regulation of expression and molecular evolution of mammalian multigene families coding for ssDNA-binding lactate dehydrogenase proteins are being studied to understand the role of the several polypeptide domains in the function and evolution of these proteins.

OBJECTIVES: In mammals lactate dehydrogenase isozymes A_4 (muscle), B_4 (heart) and C_4 (testes) are encoded by three different gene loci and the expression of these three LDH genes is developmentally regulated and tissue-specific. Recently, the LDH isozymes were shown to be ssDNA-binding proteins. The detailed knowledge about the genomic organization of this multigene family will shed light on molecular aspects of their regulation of expression and molecular evolution.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The genomic clones of three LDH genes from human and mouse have been/will be isolated and their organization and regulation of expression will be illustrated. Analysis of the DNA sequences encoding the proteins and those flanking the structural genes will allow accurate evaluations of genetic mutation events which perturb the regulatory mechanisms as well as those which modify the protein coding sequence itself. In humans the deficiencies of LDH-A or LDH-B subunits have been correlated with different clinical features and the molecular nature of their genetic mutations will be determined.

The dinucleotide CpG deficit appears to be a distinctive feature of mammalian DNA and the methylated CpG sequences appear to control the tissue-specific gene expression. The extent of DNA methylation at the regulatory region of the human LDH genes will be examined by the genomic blotting of placenta and sperm DNAs. The methylated CpG sequences have also been suggested to be mutational hotspots in mammalian DNA and the high frequency of polymorphism at Msp I and Tag I restriction sites containing the CpG sequence have been observed in human DNA.

The expression of the LDH genes may be regulated by the stress factors such as heat-shock, anaerobic-shock (anoxia, hypoxia), oxidative inhibitors, oxidizing chemicals, etc. The cellular level of nucleotide pool such as ATP/ADP, NAD+/NADH, AppppA, etc. may play an important role as signaling 'alarmones'. Recently, LDH-K isozyme is found in many carcinomas and in cultured cells transformed by the Kirsten murine sarcoma virus. The expression of this LDH-K seems to be induced by anaerobic shock. The molecular basis for the expression of the LDH-K and its possible relationship to the cancer will be studied.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: Several mouse LDH-A cDNA clones have been isolated and characterized. Mouse pMLA73 clone was found to contain the 393bp of protein-coding sequence and 496bp of the 3' untranslated region including a poly (A) tail. The amino acid sequence deduced from its open reading frame is in agreement with the COOH-terminal sequence of residues 201 to 331 of mouse LDH-A subunit determined independently by protein sequencing. This cDNA clone was used as a probe to isolate nine genomic clones from a mouse gene library. One of these genomic clones was shown to possess an insert of 13Kb.

The authenticity of mouse LDH-A functional gene and the polarity of its transcription were demonstrated by nucleotide sequencing. The exon-intron junction sites have thus far been found at amino acid no. 45, 83, 197, 236 and 278. The complete nucleotide sequence of the last exon for COOH-terminal and 3' untranslated region including the polyadenylation signal have been determined. The alternating sequence of purine and pyrimidine in the Z-DNA and two Alu-sequences have been found in the intron regions of this functional LDH-A gene and their possible roles in the regulation of gene expression will be further studied.

Eight LDH-A cDNA clones have been isolated and characterized from a human fibroblast cDNA library. Four cDNA clones appear to contain nearly full-length inserts and the complete nucleotide sequence of 1710bp consists of the protein-coding sequence (999bp), the 5' (97bp) and 3' (565bp) untranslated regions and poly(A) tail (49bp). These human LDH-A cDNA clones which were isolated from the SV40-pBR322 based recombinants will permit the functional expression of nearly full-length cDNA sequences in mammalian cells. Thus, they will be extremely useful for studying the relationship of the structure to diverse activities, including ssDNA binding of LDH-A protein, through the in vitro directed mutagenesis. Seventeen LDH-A like clones have also been isolated and characterized from human genomic library. The nucleotide sequence of a processed, intronless LDH-A pseudogene was determined. A comparison between human LDH-A cDNA and the processed pseudogene sequences reveals 114 transitions, 65 transversions and 36 deletions/insertions. Only four out of the 25 CpG dinucleotides present in the cDNA sequence remain unchanged, although the sequences possess 87.1% homology. Further, one of the human genomic clones has also been shown to possess introns and it may be a functional LDH-A gene.

Further restrictions mapping and partial nucleotide sequencing of nine mouse genomic clones and seventeen human genomic clones as well as Southern blot analysis of mouse liver DNA and human placenta DNA appear to suggest the existence of many LDH-like pesudogenes, and this may have significant implications in the mechanisms of their retroviral origin and molecular evolution.

FUTURE RESEARCH PLANS: 1. The genomic clones of LDH-A, B and C functional genes from human and mouse will be isolated and their structure determined by analyses of nucleotide sequence, R-loop and heteroduplex. The potential control (i.e. enhancer) and protein-coding sequences of different LDH genes will be compared and analyzed in order to obtain the information on genetic regulation and molecular evolution.

2. The molecular basis of human LDH-A and LDH-B deficiencies will be investigated by genomic blot analysis using LDH gene probes and, possibly, by sequence analysis of cloned mutant genes. The molecular nature of spontaneous and induced missense mutations at mouse LDH-A gene locus will also be determined.

3. The developmental and tissue-specific expression of mouse LDH-A, B and C genes will be studied by quantitative analysis of their mRNAs using gene-specific probes. The in vitro cell-free transcription system and the in vivo gene expression in cultured cells for human and mouse LDH genes will be

developed. The in vitro mutagenesis of LDH genes will be used to study the control of gene expression as well as structure-function of LDH proteins.

4. The extent of CpG methylation at the LDH-A gene will be compared among the DNA samples isolated from different tissues of human and mouse. The nuclease sensitivity of chromatin, especially, the 5' upstream regulatory region of the LDH-A gene, will be studied. In view of its ssDNA-binding ability, the possibility of autoregulation at the transcription and translation levels of LDH genes will be investigated.

5. The precise locations of LDH gene loci, especially LDH-C gene, in human and mouse chromosomes will be determined by in situ hybridization and/or genomic blot analysis of hybrid somatic cells. The possible relationship between the expression of LDH genes and oncogenes (H-ras, K-ras & N-ras) will be investigated.

PUBLICATIONS

Shimuzu, M., Tsujibo, H., Sharief, F.S., Tiano, H.F., Pan, Y.-C.E., Jungmann, R.A., and Li, S.S.-L.: Sequence analyses of cDNA and protein for mammalian lactate dehydrogenase isozymes. Fed. Proc. 42: 1970, 1983.

Tsujibo, H.,Yagi, K., Tiano, H.F., and Li, S.S.-L.: Cloning and characterization of lactate dehydrogenase genes from mouse and human. Fed. Proc. 43: 1723, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60111-05 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Role of Gene 43 DNA Polymerase in Frameshift Mutagenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.8	2	1.8

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our ongoing investigation of frameshift mutagenesis in the T4 rII system reveals the specific enhancement of particular pathways of spontaneous frameshift mutagenesis by different T4 DNA polymerases. Our studies now permit us to classify certain frameshift mechanisms by the genetic outcome (the nature of the DNA sequence change) and thus to examine the role of DNA polymerase defects exhibited by mutator and antimutator T4 DNA polymerases. For example, the mutator polymerase tsL98 produces a large increase in frameshift mutations occurring in runs of A:T base pairs (100 to 200-fold) while producing no increase in the addition of single base pairs not occurring in mononucleotide runs. Taken as a whole, our data strongly support the notion that frameshifts of these two types occur via different mechanisms.

Sequences of spontaneous frameshifts offer support for two novel mechanisms of frameshift mutation (Ripley, 1982; Ripley & Glickman, 1983; de Boer & Ripley, 1984). The first mechanism depends upon the correction of imperfect palindromic DNA sequences to more perfect palindromic DNA sequences. The second mechanism generates frameshifts by converting an imperfectly homologous sequence to a sequence that is perfectly homologous, but lies 256 base pairs down stream. This intragenic conversion event occurs at a substantial frequency in certain DNA polymerase mutant backgrounds examined. Sequences of proflavin-induced frameshift mutation in a wild type polymerase background demonstrate strong sequence specificity. Our initial results identify 14/16 mutants lie within a six base pair sequence. Not all of the mutations are identical (4 distinct genotypes have been observed). This demonstrates a direct sequence effect on occurence of frameshift mutations but may suggest the possibility of additional factors that define the specificity of these frameshift sites. Further studies in different mutant backgrounds and with other intercalating agents should permit a distinction between elements of site specificity dependent on the site of preferred mutagen binding from site specificity dependent upon site-specific DNA metabolic events.

METHODS EMPLOYED: Standard genetic manipulations of bacteriophage T4, using mutants in the rII genes to measure mutation rates and frequencies (Ripley and Shoemaker, 1983). Mutations in the DNA polymerase gene 43 were utilized to alter replication fidelity (Ripley, 1982 for review). Sequencing of T4 rII frameshifts has been accomplished by the shot gun cloning of T4 TaqI fragments in M13 followed by dideoxysequencing. In vitro exploration of frameshift fidelity has used specially constructed T4-M13 hybrid clones that permit the specific identification of frameshift mutations in T4 rII sequences in M13 DNA.

MAJOR FINDINGS AND PROPOSED COURSE: A substantial fraction of spontaneous rII frameshift mutants previously characterized genetically (Ripley and Shoemaker, 1983; Ripley, et al. 1983) has now been sequenced. Clear patterns of frameshift mutations promoted by different DNA polymerases have emerged suggesting that different aspects of DNA metabolism are altered in the various DNA polymerase backgrounds. This conclusion is based on the finding that particular "classes" of frameshift mutations are inhanced in particular DNA polymerase backgrounds. The patterns of enhancement suggest that although one class may be enhanced by a particular polymerase, similar enhancement of all classes does not occur. A simple conclusion from this observation is that frameshift mutation frequencies do not occur at enhanced frequencies in mutant DNA polymerase backgrounds as a general consequence of poor DNA replication, but instead occur in a specific manner implicating the active participation of the mutant DNA polymerase in the initial production and/or the final fixation of the frameshift mutation.

Classes of frameshifts:

(1). The addition or deletion of a single base pair in a run of identical base pairs. (e.g. 4 consecutive A:T pairs are mutated to either 5 or 3 consecutive A:T pairs.)

A. These mutations are extremely frequent in tsL98 and tsL56 polymerase backgrounds, but are infrequent in the tsL141 background when compared to the wild type DNA polymerase. This result might suggest that proofreading of the mismatched mutational heterozygote might be a major factor in the frequency of this class of frameshift mutation since the tsL98 and tsL56 polymerases demonstrate relatively low 3'-5' exonuclease activity in vitro, while tsL141 exhibits relatively high exonuclease activity compared to the wild type.

B. Mutations of this class are also enchanced in a tsL88 DNA polymerase background. Since this mutator polymerase does not exhibit altered proofreading, the enhancement of this class of frameshift mutations in this background probably occurs by an alternative mechanism.

(2). The addition or deletion of multiple base pairs as a consequence of two nearby homologous or nearly homologous DNA sequences.

A. The most frequently recovered mutation of this type in our DNA target results in the addition or deletion of 8 bp within the sequence 5'-ATTGGCTGATTGGC-3'

leaving either 5'-ATTGGCTGATTGGCTGATTGGC-3' or 5'-ATTGGC-3'. Such a mutation is likely to arise through a misalignment of one copy of the ATTGGC sequence on the complement of the other copy, the direction of misalignment relative to DNA synthesis determines whether the outcome is + or -8 bp. We detect no large difference in the frequency of additions versus deletions in any DNA polymerase background.

B. The frequency of enhancement of this class of frameshift mutation is significant in the mutator DNA polymerase backgrounds, but is substantially smaller than the addition or deletion of single bases in runs of that base described above. For example, tsL98 increases the frequency of the single base addition or deletions by 100 to 200-fold, while increasing the frequency of + or -8 bp in the target described here only 3 to 7-fold.

C. Other deletions or duplications have been found to correlate with the presence of repeated sequences at their end points. In some cases these repeats are not perfectly homologous. In one deletion the repeat has 12 of 17 homologous base pairs.

(3). The duplication of single base having nearest neighbors that are different from that base. (e.g. the duplication of a G in the sequence 5'-AGC-3').

A. This class of frameshift is not explained by misalignments in which a misaligned, but Watson-Crick base paired primer can be offered as a substrate for DNA synthesis. Approximately 1/3 of all single base additions detected in the wild type DNA polymerase background were of this type.

B. This class of frameshift was not enhanced by tsL56 or tsL98 mutators, but was seen in the tsL141 background.

C. The sequences in which these mutations arise most frequently appear to bear some homology that extends past its nearest neighbors. For example 6 out of 8 base pairs are identical between two sites at which mutations have been detected. The same mutations are not detected in any of three other sites in the DNA target that offer identical nearest neighbors. The potential for sequence specific effects to offer clues as to the mechanism responsible for these mutations is being examined. For example, do these sites correlate with in vitro pause sites exhibited by T4 DNA polymerase in this DNA sequence?

(4). Complex sequence changes, frameshifts associated with other base pair changes.

A. Quasipalindrome directed mutation. Attempts to recover such mutants at high frequency in the large DNA target examined to date have not been successful. A smaller target is to be constructed which should produce a much higher relative frequency of these mutations. Additional power for detection is being developed by using oligonucleotides to probe directly for the complex DNA sequence change.

B. Conversion events directed by imperfect small homologies. The clearest case for these mutations can be made in the generation of a mutant -rFC47- where a TT dinucleotide sequence is replaced by a CTG trinucleotide sequence. The sequence responsible is likely to be one lying 256 bp downstream, which is otherwise homologous to the sequence surrounding the TT dinucleotide for a distance of 16 additional bp. Mutations of this type have been recovered in tsL88, tsL141 and wild type polymerase backgrounds. We intend to demonstrate that this downstream sequence is indeed responsible for the mutation by site-specific alteration of that sequence to demonstrate the predicted altered frame-shift mutation. At least one other instance of this mechanism may well be responsible for two mutations occurring in the tsL141 polymerase background. The sequence change in that case results in the replacement of 5 base pairs with 7 others. The source of that sequence however, remains unknown. We intend to probe T4 and host DNA to identify a potential source. This mechanism of mutagenesis may well be responsible for a large class of tsL141 induced mutants, since 4 of a sample of 48 mutants were the FC47 mutation.

C. Miscellaneous complex mutations that do not fall in the upper two classes, are largely identical in their topology of having a frameshift separated by either two or three base pairs from a transversion (in one case a transition). All are isolated as single instances, and there is little overlap in the sequences. 8 separate isolates in three polymerase backgrounds have been found to date. This number should be increased soon as additional spontaneous fs mutant sequences from a wild polymerase background are determined in clones that have already been isolated. Here we are attempting an in vitro approach by offering T4 DNA polymerase a mismatched primer already bearing a transversion mismatch, followed by two good base pairs in hopes that such an intermediate might stimulate the production of a frameshift. (The general model is that per-turbation in the DNA polymerase binding to template DNA causes the downstream mutations. This model might be generalizible to the production of mutation by bulky adducts as a consequence of DNA alkylation.)

(5). Duplication or deletion of dinucleotide DNA sequences.

A. Eleven such sequences have been found. In only one case does a dupli-cation lie in a repeat (3C's go to 5C's). The remaining examples are AA, AG, AT, CT sequences that are not in repeats. A high fraction of these mutations lie at the base of potential DNA hairpins. One hypothesis is that such muta-tions occur as a consequence of interaction of such a structure with DNA repli-cation in such a manner that stuttering of the polymerization reaction is enhanced. In one test we are examining the fs sequences surrounding a newly introduced DNA hairpin to determine whether this hairpin will produce such dinucleotide deletions or additions.

(6). Single nucleotide insertions.

A. Frameshift mutations involving added bases detected to date probably arise primarily by duplication. Although there are some exceptions among the

complex frameshift mutations. Were insertions common and random, they would be expected to be recognized between 50% and 75% of the time. Although such insertions can be common in particular polymerase backgrounds, they do not arise throughout the DNA target, but instead have been limited to date to two sites. At one site the insetion of a T (and in one instance the insetion of a C) has been recovered repeatedly in the sequence 5'-TTG()AAA-3'

T
C

Five instances of this event were recovered in the tsL141 background which may suggest that it is a conversion event like that generating FC47. No simple nearby palindrome predicts the change. The single instance of an insertion of a C at this site (in a tsL88 background) makes the issue complex. Is the event templated? or is it the semi-random incorporation of a base in which the incorporation reaction favors T over C. Only one other single base insertion has been recovered--an insertion of a G in the sequence AC()AC--this insertion in a tsL98 Polymerase background.

(7). Frameshifts enhanced by a particular polymerase.

A. An exceptional frameshift hotspot was noted in the tsL141 polymerase background. Despite a general decrease in frameshifts occurring at run's of A:T base pairs in this genetic background, the deletion of a T in a sequence 5'-ATTGGC-3' was found 16 times in a sample of 48, representing an increase in frequency of this mutation over the wild type polymerase background of about 200-fold. The sequence responsible for this highly specific frameshift enhancement lies beyond that indicated above however, since no instances of this mutation were discovered in the same sample in an identical ATTGCC sequence lying 8 base pairs upstream. This strongly specific enhancement suggests the action of novel mechanism in this particular DNA sequence.

SPECIFICITY OF PROFLAVIN ENHANCED FRAMESHIFT MUTAGENESIS.

Intercalating dyes such as acridines specifically enhance frameshift mutation frequencies. We have set out to determine the type of frameshift mutations that are enhanced by the mutagen proflavin as a clue to the specific mutational mechanism responsible.

Our initial results indicate the strong propensity of frameshift mutations to cluster in a sequence 5'-TGTTAAAC-3' among 16 mutants sequenced to date, 14 lie at this site. The addition of G and of A but of no other bases are frequent. The deletion of a T between the G and the A's has been found as well as the duplication of TA. With the exception of the addition of an A, these mutations have not yet been detected among hundreds of spontaneous mutations sequenced to date, suggesting a very specific response to the presence of proflavin.

Identification of the recognition sequence responsible for this hot spot will be undertaken by examining mutants containing duplications of other small DNA

sequence changes in this region. Association of the hot spot with the polymerase specificity will be tested by examining the specificity of proflavin induced mutation in a mutant DNA polymerase background. Absence of such an association would suggest that the primary cause of frameshift mutagenesis by this mutagen is attributable to the mutagen DNA complex itself and thus to be expected in this sequence. A test of this hypothesis can be undertaken in this exact DNA sequence in M13 DNA replicated in vivo by M13 replication system or in vitro. Absence of a demonstrable frameshift hotspot in these systems would implicate some other enzymatic system in this hotspot. An attractive candidate would be other T4 replication proteins. Because the site predominates so dramatically, only a small sample of mutant sequences will be needed to probe for altered specificity in other mutant backgrounds. An attractive candidate is the T4 topoisomerase (gene 39, 58, 60) which can be inhibited in vitro with AMSA an intercalating anti-tumor agent.

C. Publications:

Ripley, L.S.: Model for the participated of quasipalindromic DNA sequences in frameshift mutation. Proc. Natl. Acad. Sci. USA 79: 4128-4132, 1982.

Ripley, L.S.: The specificity of infidelity of DNA polymerase. In Lawrence, C.W., Prakash, L.S., and Sherman, F. (Ed.): Induced Mutagenesis: Molecular Mechanisms and Their Implications for Environmental Protection. Plenum Press, NY, 1982, pp. 83-113.

Ripley, L.S., and Shoemaker, N.B.: Polymerase infidelity and frameshift mutation. In Lemontt, J.F., and Generoso, W.M. (Ed.): Molecular and Cellular Mechanisms of Mutagenesis. Plenum Press, NY, 1981, pp. 161-178.

Ripley, L.S., and Glickman, B.W.: The unique self-complementarity of palindromic sequences provides DNA structural intermediates for mutation. Cold Spring Harbor Symp. Quant. Biol. 47: 851-861, 1982.

Ripley, L.S., and Shoemaker, N.B.: A major role for DNA polymerase in frameshift mutation. Genetics. 103: 353-366, 1983.

Ripley, L.S., and Glickman, B.W.: DNA secondary structures and mutation. In Friedberg, E.C. and Bridges, B.R. (Ed.): Cellular Responses to DNA Damage, UCLA Symposia on Molecular and Cellular Biology, A New Series, Alan R. Liss, NY 11: (in press), 1983.

Ripley, L.S., Glickman, B.W., and Shoemaker, N.B.: Mutator versus antimutator activity of a T4 DNA polymerase distinguishes two different frameshifting mechanisms. Mole. Gen. Genet. 189: 113-117, 1983.

Glickman, B.W., and Ripley, L.S.: Structural intermediates of deletion mutagenesis: a role for palindromic DNA. PNAS. 81: 512-516.

Drake, J.W., Glickman, B.W., and Ripley, L.S.: De mutatis mutationum mutandis. Amer. Scientist. 71: 621-630.

de Boer, J.G. and Ripley, L.S.: A demonstration of the products of frameshift and base substitution mutation by quasipalidromic DNA sequences. PNAS (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60113-05 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Mechanisms of Mutagenesis in E. coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. W. Glickman	Expert	LGM, NIEHS
	R. M. Schaaper	Visiting Fellow	LGM, NIEHS

COOPERATING UNITS *(if any)*

Dr. R. Fowler, Department of Biology
San Jose State University, San Jose, CA

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 60149-01 LG entitled "Molecular mechanisms of mutation: Mutational Specificity"

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60130-03 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation of E. coli Mutants Defective in Repair of Alkylated DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. R. Volkert Senior Staff Fellow LGM, NIEHS

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 60150-01 LG entitled "Gene Induction by Alkylation Treatments in E. coli"

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60132-04 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interaction of recF143 and recA441 Mutations in DNA Repair and Mutagenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. R. Volkert Senior Staff Fellow LGM, NIEHS

COOPERATING UNITS *(if any)*

Alvin J. Clark and Linda J. Margossian
Department of Molecular Biology, Univ. of California
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 60151-01 LG entitled "Role of the recF Gene in DNA Repair"

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60136-03 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultraviolet Mutagenesis in Bacteriophage T4

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: J. W. Drake Head LGM, NIEHS

COOPERATING UNITS *(if any)*

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NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 60148-01 LG entitled "Error-Prone Repair in Bacteriophage T4"

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60143-02 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Specificity of Mutagenesis in Mammalian Cells Using Cloned Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. A. Zakour	Senior Staff Fellow	LGM, NIEHS
	B. W. Glickman	Expert	LGM, NIEHS
Others:	R. M. Schaaper	Visiting Fellow	LGM, NIEHS
	E. Drobetsky	Guest Worker	LGM, NIEHS

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to examine at the molecular level the specificity of mutagenesis in mammalian cells. To do this, we are developing a system using cloned DNA sequences that can be shuttled into and out of the eucaryotic chromosome. The use of the cloned gene for thymidine kinase (TK) from Herpes Simplex Virus provides a defined target of known DNA sequence in which a broad range of mutagenic events, including nucleotide substitutions, frameshifts and small additions or deletions, can be examined. The specific aims of this project are to establish an experimental system in which this can be accomplished and to initiate an examination of the mutational specificity of a variety of potential environmental mutagens as well as the molecular nature of spontaneous mutagenesis in mammalian cells.

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to develop a system for the repeate determination of the exact nature of mutational changes at the level of DNA sequence in mammalian cells. This will require the recovery and cloning of a ger tic target that has been mutated in the cell. The use of E. coli plasmid vectors will allow for the screening and DNA sequence analysis of each mutant. In order do this the target gene must be able to be expressed in both the mammalian and ba terial cells. The shuttle system entails the insertion of a specific gene into a defined DNA context into a mammalian chromosome, the recovery of these genes from the mammalian cell, and their recloning into E. coli for molecular charac- terization.

METHODS EMPLOYED: CONSTRUCTION OF THE SHUTTLE VECTOR: A composite vector carryi the thymidine kinase gene and galactose kinase gene was constructed in two steps. Plasmid pSVK carries the E. coli galactokinase gene (GK) under control of the SV4 early promotor (a gift of M. Rosenburg). A small fragment (EcoRI-BamHI) of pSVK was replaced by the EcoRI-BamHI fragment carrying the Tet promotor of pBR322 yielding pRS. The plasmid pKOS17B2 carrying the TK gene (obtained from P. Schaffer) was used as the source of a 2800 base pair BamHI-BglII fragment carryin the TK gene (but not its HSV promotor). This was inserted into the unique Bam si of pRS in the orientation permitting the Tet promotor to function for the TK gene In this orientation the TK gene is expressed in both E. coli and mammalian cells (our own experiments and Kit et al 1981). This plasmid has been named pGK/TK. We have also used a multi-gene plasmid system which carries the gene for resistan to the antibiotic G418 (a gift of S. Larson) in addition to the TK and pBR322 sequences indicated above (pTK/G418). The use of resistance to G418 provides a dominant selectable marker in mammalian cells. We have demonstrated the expressi of this gene in mouse L cells that have received the plasmid via transfection.

METHODS OF CELL CULTURE AND MUTANT SELECTION: The TK^- mouse L cell line, B6, is grown in Delbecco's modified Eagle's medium supplemented with 10% fetal calf seru and antibiotics. For the selection of TK+ lines the growth medium was supplement with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.7×10^{-5} M thymidine (HAT medium). For the selection of G418 resistant lines, the medium is supplemented with 400-800µg/ml of G418 (Gibco). TK mutants are isolated by their resistance t trifluorothymidine (1µg/ml).

RECOVERY OF MUTANT DNA: Prior to its insertion into the host chromosome the plasmid is cut with a restriction enzyme that makes a single cut in the plasmid a ligated to carrier DNA cut with the same enzyme (e.g., BamHI in the case of pGK/T or HindIII for pTK/G418). Following the isolation of the mutant cells, DNA con- taining the target sequences is treated with the same restriction enzyme. The resulting material is then ligated under conditions favoring the formation of circles and used to transfect E. coli by selecting for ampicillin resistance usin recently devised high efficiency bacterial DNA transformation protocols. We have found that an excess of mammalian DNA (100,000:1) only reduces the transforming efficiency of the plasmid pBR322 by 50%. Should this method for the recovery of mutated TK genes prove too inefficient, several alternative approaches are available. The in situ plaque hybridization assay of Benton and Davis (1977) allows the recovery of bacteriophage clones bearing specific gene sequences from

large "random" libraries of phage born DNA. However, in a typical application of this assay, as many as 10 clones may be screened simultaneously for the presence of a specific sequence. This is not only tedious, it is also a rate limiting step. Alternatively, the method devised by Seed (1983) may also be tried to recover by in vivo recombination the genes from Lambda DNA libraries prepared from the mutant cells.

For the purpose of mapping, we have cloned the HSV-TK gene into pBR325 and it is in this plasmid that the deletion mutants will be constructed. In vivo recombination requires that both the mutant and the deletion plasmids be grown together. This is possible since the deletion mapping plasmid carries a CAM-resistance marker and the mutant plasmid, an ampicillin-resistance marker.

DNA SEQUENCING: Well designed sequencing strategies permit the sequencing of large numbers of mutants. This is aided by the fact that both spontaneous and induced spectra reveal that a large majority of mutations occur at a small number of sites (i.e. hotspots). In addition, the genetic localization by recombinational analysis will greatly facilitate the sequencing process.

For the DNA sequence analysis, we will reclone the mutant TK DNAs into an M13 derivative, e.g., WB2344 (Barnes and Bevan 1983) and sequence using the "dideoxy" chain terminator method (Sanger et al 1977, Sanger and Coulson 1978, Smith 1980). Using synthetic primers (14-mers) spaced about every 100 nucleotides along the TK DNA sequences any mutant can be readily sequenced efficiently and accurately. We are currently applying this method of DNA sequencing to the routine analysis of lacI mutations.

CHOICE OF MUTAGENIC TREATMENTS: Besides studying the specificity of spontaneous mutagenesis, we intend to determine the mutational specificity of a number of carcinogens important in the environment as well as several mutagens currently used in medical treatment. These include ionizing radiation, UV irradiation, ethyl methanesulfonate, benzo(a)pyrene, and N-acetoxy-2-aminofluorene.

To date, we have focused our efforts on the establishment of a reproducible experimental system in which the repeated determination of the exact nature of mutational changes in mammalian cells can be accomplished. We have examined a variety of approaches for the insertion and recovery of cloned genes in several different types of eucaryotic cells and have found suitable genetic and cellular systems for further experimentation. We are presently working on the characterization of cell lines that have received the cloned genes and on the methods of recovery of these genes from eucaryotic cells. Then studies of the effects of specific mutagens will be initiated. The production of mutational spectra will be a demanding process and it is recognized that the proposed spectra can only be completed over an extened time period. However, within the next year, the system should be developed and modified to the point where mutational spectra are being produced on a routine basis. Some indications of the relative roles of base substitutions, deletions, additions and frameshifts in mutation in mammalian cells should be available.

This project will be terminated at NIEHS in the autumn of 1984 since all investigators will be leaving the Institute.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This proje provides a molecular approach to mutagenesis in mammalian cells. The availabilit of spontaneous and induced spectra from mammalian cells will provide important clues to the mechanism of mutation in mammals. Such studies may also provide ind cation of the types of mechanism involved in the repair of lesions in cells and thus may be important in the elucidation of manner in which cells respond to environmental agents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 60145-02 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Mutational Specificity of Purified DNA Replication and Repair Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. A. Kunkel	Senior Staff Fellow	LGM, NIEHS
Others:	J. C. Liu	Biologist	LGM, NIEHS
	J. L. Motto	Biologist	LGM, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Genetics

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INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	0.8	1.8

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mutational specificity of highly purified and well characterized DNA replication and repair proteins is being examined in in vitro DNA synthesis reactions using biologically active viral DNA templates. Both a forward mutational assay capable of detecting a wide spectrum of errors (base substitutions, frameshifts, deletions, etc.) in M13 mp2 DNA, as well as highly specific reversion assays, are being employed. The work is presently focused on the accuracy of the proteins primarily responsible for the synthesis of new DNA, the DNA polymerases themselves. The accuracy of the three classes of eucaryotic DNA polymerases, established in the forward system, are very different; the mutation frequencies, per round of DNA synthesis in vitro are: polymerase -γ, 40 X 10^{-4}; polymerase -α, 80 to 200 X 10^{-4} and polymerase-β, 400 to 800 X 10^{-4}. DNA sequence analysis of over 1000 mutants indicates dramatic and informative differences in the kinds of mutations produced. For example, frameshift mutation frequencies are 300 X 10^{-4} for Polβ and <1.0 X 10^{-4} for Polγ. This correlates with the "processivity" of these enzymes. Comparably large differences in specific base substitution errors are also observed. These experiments are intended to provide detailed information on the parameters of protein-nucleic acid interactions which are important in determining accuracy. The studies are being expanded to employ prokaryotic DNA polymerases capable of proofreading, as well as additional "accessory" proteins from prokaryotic and eukaryotic systems.

A. Research Project:

Problem: Our understanding of the mechanisms used by a cell to replicate and maintain genetic information with high accuracy is incomplete. Even less well understood as the mechanisms which generate the many mutational responses resulting from perturbation of these processes.

Objective: This project addresses two general and very much interrelated issues. 1. What is the contribution of each of the putative replication and/or repair proteins to accuracy? 2. At the level of protein-nucleic acid interactions, what mechanisms are operative to achieve this accuracy?

Scientific Justification and Experimental Approach: The current gaps in our knowledge of fidelity determinants stem in part from the lack of a system to address questions of frequency and specificity in a manner which will allow a determination of the exact nature of these rare events and at the same time permit analysis of mechanisms at the level of protein-nucleic acid interactions. The proposed experiments are intended to provide such information for the crucial enzymes involved in the synthesis of the genetic information. Such information is necessary for understanding mutagenesis at the molecular level. A determination of the cellular mechanisms for achieving the accurate production and maintenance of the genetic information is essential to understanding several fundamental biological processes.

Highly purified DNA replication and repair proteins are used to synthesize a DNA strand in vitro under defined reaction conditions, using as a template the single stranded circular DNA from either M13mp2 or oX174 phage. The DNA sequence which is the target for mutagenesis can be chosen to analyze specific errors, or a broad spectrum of errors, including base substitutions, frameshifts, deletions or duplications. The newly synthesized compelentary strand, containing the mutation to be analyzed, is expressed by transfection of the product of the in vitro reaction into competent E. coli cells. Mutants are selected on the basis of plaque color (M13mp2) or ability to grow on suppressor minus bacteria (oX174), and are analyzed by DNA sequencing.

Recent Accomplishments: The M13mp2 forward mutational assay has been used to establish the accuracy of several representatives of each of the three major classes of mammalian DNA polymerases (α, β and γ). These enzymes have been analyzed first because they are of eukaryotic origin and lack 3'→5' exonuclease proofreading activity, making interpretation of mechanisms less complicated. DNA sequence analysis of more than 1000 mutants (the major effort of the past year) indicates substantial differences in mutational specificity for these enzymes. The frequency of single base deletion frameshift errors (Pol β, 300 X 10^{-4}; Pol-α, 25 X 10^{-4}; Pol-γ, <1.0 X 10^{-4}) correlates well with the ability of the enzyme to remain associated with the primer template during polymerization, i.e. processivity (Polβ, 1; Polα, 10 to 20; Polγ, >1000 nucleotides added per association event). Thus processivity, whether in vitro with a purified protein or perhaps in vivo in a replication complex, seems to be an important property in determination of frameshift mutations. Similarly large differences are observed in the frequency of specific base substitution errors for each enzyme.

Completion of a more extensive set of mutant sequences will provide information on the relative importance of base hydrogen bonding, stacking interactions and the actual DNA polymerases themselves in determining base substitution errors.

In the last two months two sets of mutations have been discovered, produced by DNA polymerase β, a putative repair enzyme in vivo, which may prove instructive in future studies. One is a set of frameshift and base substitution mutations which suggest a novel mutagenic mechanism which is currently being examined. The other is a set of specific deletion muations which may provide insight into the properties of both the primer template and enzymes which are important in defining this class of mutational events.

Preliminary results with prokaryotic DNA polymerases containing associated 3'→5' proofreading exonuclease activities suggest that the forward mutation assay will be useful in determining the role of proofreading in correction of various types of errors.

B. Plans for Subsequent Year:

Two categories of experimentation will be pursued in the next two to three years, in order to directly address the two questions posed in "Objectives". The first is to continue to utilize the forward mutational assay to assess the frequency and specificity of mutations produced in reactions using defined protein components. Emphasis will continue to be on DNA polymerases for at least one more year and possibly more, since the new system in use is powerful and too recent to have been fully exploited as yet. The role of accessory proteins in improving accuracy will also be examined. The expectation is that such accessory proteins will affect the DNA structure (DNA binding proteins) polymerase processivity (holoenzyme subunits), proofreading (3'→5' exonucleases) and/or perhaps substrate (dNTP) binding affinity. The second and related set of experiments addresses questions of mechanisms, and will use very specific mutational targets, most often in reversion assays. Two examples of this are currently in progress. The first is to determine the effect of directly altering processivity on frameshift mutation frequency. The second is to directly test a novel slippage and realignment model for base substitution mutations observed at a DNA polymerase-β hotspot in M13mp2. Both reversion and forward mutational assays will be used to examine in detail the specificity and mechanisms of proofreading, and to search for putative proofreading activity in eukaryotic systems.

C. Publications of Past 18 Months:

Das, S., Kunkel, T.A., and Loeb, L.A.: Altered nucleotide pools as a basis of mutagenesis. In de Serres, F.J. (Ed.): Genetic Consequences of Nucleotide Pool Imbalances. New York, Plenum, 1984, (in press).

Kunkel, T.A., Loeb, L.A., and Goodman, M.F.: On the fidelity of DNA replication. The accuracy of T4 DNA polymerases in copying oX174 DNA in vitro. J. Biol. Chem. 259: 1539-1545, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60146-02 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mutagenic Consequences of Defined Lesions in DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. A. Kunkel	Senior Staff Fellow	LGM, NIEHS
Others:	J. C. Liu	Biologist	LGM, NIEHS
	J. L. Motto	Biologist	LGM, NIEHS

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The exact mutagenic consequences of introduction of defined lesions into DNA are being examined in a forward mutational system using M13mp2 DNA and capable of detecting a wide spectrum of mutational events, both at and some distance away from the actual site of damage. The system has first been applied to depurination, the loss of a purine base from DNA. This is a frequent spontaneous lesion as well as a common intermediate in the repair of many other types of DNA damage. Depurination is highly mutagenic, as determined by transfection of depurinated M13mp2 DNA into SOS-induced compentent E. coli cells. DNA sequence analysis of 211 mutants demonstrates that most mutations are base substitutions reflecting insertion of dAMP opposite the non-coding abasic site and resulting in characteristic transversions. In order to determine the effects of a single abasic site placed at a defined position in the DNA, a general approach has been developed which should be applicable to many diverse types of premutagenic lesions. This procedure utilizes uracil containing DNA templates for standard in vitro reactions to incorporate the lesion into a covalently closed circular, and thus biologically active, molecule. The product of this reaction is then treated to yield, as the sole source of biological activity, complementary strand circles containing to lesion of interest at a single site and at essentially 100% frequency. In addition to its utility in studying lesions in DNA, the technique can be applied to standard site specific mutagenesis protocols to obtain very high efficiency even without selection.

A. Research Project:

Problem: Defining the mutagenic potential of DNA damaging agents is complicated by the fact that most agents produce a spectrum of different lesions at multiple positions and varying frequencies and because several mutational outcomes are possible.

Objective: To define exact mutational endpoints for specific lesions in DNA. The first lesion examined is the abasic site, resulting from depurination, the loss of a purine base from the DNA.

Scientific Justification and Experimental Approach: These studies are intended to provide detailed information on the exact mutagenic potential of specific lesions in DNA. This information is critical to our understanding of the risk posed by various DNA damaging agents. The abasic site is the first lesion to be examined for two reasons. It is by definition a non-coding lesion and has been estimated to occur at high frequency both spontaneously and as a common intermediate in repair of many other DNA lesions.

Mutagenicity is examined using the single-stranded bacteriophage M13mp2, which contains the α-peptide coding region of the lacZ gene of E. coli. Mutants obtained from a transfection assay using normal or depurinated DNA are scored as having a light blue or colorless phenotype compared to wild type blue color, when plated on the appropriate indicator plates. The exact nature of the mutational events is then determined by chain terminator DNA sequence analysis.

This system allows exact determination of many classes of mutational events including base substitutions, frameshifts, deletions, and duplications.

Recent Accomplishments: The mutagenic consequences of damage to DNA produced by low pH and high temperature (primarily depurination) have been determined in a forward mutational system. Transfection of depurinated single strand M13mp2 DNA into competent cells results in a 15-fold increase in the frequency of mutant (light blue or colorless) plaques compared to a non-depurinated DNA control. Mutagenicity is proportional to the number of lethal sites introduced into the DNA and is largely dependent on a functional error prone repair system in the competent E. coli cells. Approximately 90% of the damage-dependent increase in mutagenicity is abolished by apurinic endonuclease or by alkali-treatment of the damaged DNA prior to transfection. Based on these observations and the rate constants for formation of the various types of heat/acid produced lesions in DNA, it is concluded that the majority of the induced-mutagenesis results from the presence of abasic sites in the DNA. DNA sequence analysis of 87 spontaneous and 124 induced mutants indicates that three types of mutational events are increased: base substitutions (31-fold), double mutations (>33-fold) and mutations arising from recombination between the M13mp2 DNA and the complementary lac information on the F' in the host cell (16-fold). There is only a slight increase in the frequency of frameshift mutations (3.8 fold) and essentially no increase in large deletion mutations. Approximately 80% of the base substitution mutations occur at purine positions in the viral strand, consistent with depurination as the predominant premutagenic lesion. The 2:1 ratio of G:A sites mutated is consistent with the preference for depurination of G over A.

Transversions are observed for 57 of 79 (72%) induced base substitutions, with a strong preference for insertion of A opposite the putative apurinic site.

In addition to the classical approach just described, the most recent efforts have attempted to determine the mutagenic consequences of a single abasic site placed at a single position in M13mp2 DNA. In working out the details of this approach, a technique has been developed which has already proven to be very useful not only for the initial purpose of placing a lesion at a defined position, but also for rapid and highly efficient site specific mutagenesis, even without phenotypic selection. Site specific mutations have been introduced into the coding sequence of the lac Z_α gene in M13mp2 DNA at efficiencies between 40 and 90%, in a simple procedure requiring a few hours to complete. The high efficiency is obtained using uracil-containing viral DNA, which has normal template coding potential for the in vitro reactions typical of current site specific mutagenesis protocols, but which is strongly selected against upon transfection into a wild type E. coli host cell. Expression of the desired change, present in the newly synthesized, non-uracil-containing complementary strand, is thus favored. The procedure works equally well for either selectable or silent mutations, and is applicable to almost any cloned DNA sequence.

B. Plans for Subsequent Year: Because of its potential for a wide variety of applications in many fields, the site specific mutagenesis procedure will be developed in more detail. This will include construction of frameshift, deletion and insertion mutations, and attempts to improve efficiency to 100% while maintaining high biological activity. These latter two issues are crucial to the application of uracil-containing templates to specific lesions at defined sites.

The experiments to examine the mutational consequences of a single abasic site will be continued using a uracil containing oligonucleotide. The uracil will be converted to a single abasic site through the action of uracil glycosylase, and the molecules containing this lesion will be expressed by transfection into competent E. coli cells. These studies will be expanded to include several different positions for the abasic site as well as different replication and repair backgrounds. Ultimately the system will be used for other defined DNA lesions.

C. Publications in Last 18 Months:

Kunkel, T.A., Schaaper, R.M., and Loeb, L.A.: Depurination-induced infidelity of DNA synthesis with purified DNA replication proteins in vitro. Biochemistry 22: 2378-2384, 1983.

Kunkel, T.A.: The mutational specificity of depurination. Proc. Natl. Acad. Sci. USA 81: 1494-1498, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60147-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Mechanisms of SOS-Mutagenesis in Escherichia Coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: R. M. Schaaper Visiting Fellow LGM, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
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SECTION
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INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to study the molecular events that lead to mutation in the bacterium E. coli after induction of the SOS-response. The error-prone type of DNA replication that is presumed to be responsible for SOS-mutagenesis will be studied in an in vitro replication system. The accuracy with which crude extracts of E. coli cells copy normal or damaged single-stranded bacteriophage M13 DNA will be used as an indicator for in vitro SOS-expression. Characterization of the components involved is important for the study of SOS-mutagenesis and for the question of the regulation of mutation rates in general.

A. Research Project:

Problem: In the study of mutagenesis in E. coli the SOS-system plays a central role. This system, induced when damage to the DNA of the organism blocks or impedes DNA replication, is multifacetted and is believed to include an error-prone replication component responsible for mutagenesis on both intact and damaged DNA.

By genetic analysis several genes participating in this process have been identified. However, the exact biochemical nature of the mutational process has remained obscure. Elucidation of this pathway will not only benefit the study of mutagenesis but might also be important for our understanding of how organisms can regulate their mutation rates as a response to changing and challenging environments.

Objectives: We want to develop an in vitro assay-system for the error-prone-replication which occurs inside the cell upon induction of the SOS-response. This in vitro assay system will be used to disect and characterize the components of this error-prone-replicational system.

Experimental Approach: We will use the in vitro replication of the single stranded DNA-phage M13 in a crude extract of E. coli cells, converting single stranded DNA to double stranded RFDNA. This replication system is well established and its components have been identified. After replication of either normal or damaged DNA templates with extracts derived from normal or SOS-induced E. coli cells, the product DNA will be assayed for mutation frequency by transfection. In vivo experiments already have demonstrated that phage M13 is a good target for SOS-mutagenesis in this first round of replication. A corresponding in vitro response should therefore be obtainable.

Accomplishments: This project has just been started. Experimental procedures have been developed for efficient in vitro replication and recovery of replicated DNA for transfection. Current emphasis is on development of the mutational assay.

B. Publications in Last 18 Months:

Schaaper, R.M., Kunkel, T.A., and Loeb, L.A.: Infidelity of DNA synthesis associated with bypass of apurinic sites. Proc. Natl. Acad. Sci. USA 80: 487-491, 1983.

Kunkel, T.A., Schaaper, R.M., and Loeb, L.A.: Depurination-induced infidelity of DNA synthesis with purified DNA replication proteins in vivo. Biochemistry 22: 2378-2384, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60148-01 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Error-Prone Repair in Bacteriophage T4

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. W. Drake	Head	LGM, NIEHS
Others:	F. W. Coleman	Senior Staff Fellow	LGM, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Genetics

SECTION

Mutagenesis Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is an analysis of error-prone repair in bacteriophage T4. Most chemical and all radiation mutagenesis in T4 occurs via error-prone repair, and depends on the functions of the genes uvsW, uvsX, uvsY and virtually all genes required for DNA replication. We have recently obtained and characterized conditional (temperature-sensitive and amber) alleles of the X and Y genes. The ts mutants are of particular interest because they differentially affect three different traits of the mutants, namely suppression of a gene 49 (Holliday resolvase) defect, ultraviolet radiation sensitivity, and ultraviolet mutagenesis. We plan genetic tests of the effects of these mutations upon recombination (which also depends upon the WXY system), tests to ask whether recombination and mutagenesis are correlated, tests to determine which other genes of DNA metabolism are required for ultraviolet mutagenesis, tests of the sensitivity of mutants of the WXY system to photodynamic inactivation mediated by isopsoralen, tests to determine if the putative "uvsZ" gene is involved in error-prone repair, and screens for temperature-sensitive uvsW mutations. We plan to purify uvsX proteins from cells infected with ts uvsX mutants and to compare their biochemical properties with those of the wild-type protein, in order to determine which biochemical differences correspond to changes in survival versus mutagenesis after ultraviolet irradiation. Later, we plan to conduct the same kind of analysis of uvsY proteins.

A. Research Project

Most mutagenesis induced by most chemicals and radiations in most organisms occurs during the course of error-prone repair (EPR), a process operationally defined by mutants that exhibit increased sensitivities to inactivation and decreased sensitivities to mutagenesis by diverse mutagens. Analysis of the mechanism(s) of EPR has proceeded most rapidly in two microbial systems: the SOS system of E. coli, and the WXY system of bacteriophage T4. The problem of the moment is to determine which genes are involved in this mutagenic response, and how. Ultimately, the problem is how to manipulate the EPR process in ways that decrease mutagenesis but do not unduly decrease cell survival or function.

The most immediate objectives of this project are to conduct a number of complementary genetic and biochemical analyses of the T4 WXY system to determine (1) whether temperature-sensitive mutations of uvsX and uvsY differentially affect recombination in T4, as they do several other aspects of the WXY system; (2) whether recombination and mutagenesis are functionally correlated; (3) whether the full functions of other genes of T4 DNA metabolism are required for the mutagenic response in EPR; (4) whether ts mutants of uvsW can be isolated by screening for mutants temperature-sensitive for hydroxyurea sensitivity; (5) whether the relative sensitivities of WXY mutants (compare to the wild type) to isopsoralen-mediated photodynamic inactivation are similar to or greater than their relative sensitivities to ultraviolet irradiaton; (6) whether the putative "uvsZ" gene is involved in EPR; and (7) whether uvsX and uvsY proteins, purified from cells infected with the ts mutants of these genes, display differences from the wild-type proteins that correlate with their differential effects upon suppression of a gene 49 (Holliday resolvase) defect, sensitivity to UV inactivation, and sensitivity to UV mutagenesis.

These objectives all bear on the questions of the mechanism of EPR and its evolutionary significance. Most of them also reflect recent work in this laboratory (Conkling and Drake 1984a and b) in which temperature-sensitive mutants of uvsX and uvsY were isolated and characterized. Many of these ts mutants affect the three WXY functions examined to date to very different extents, indicating that diverse WXY functions can be uncoupled mutationally and suggesting that they may be affected by different domains of the uvsX and uvsY proteins. The three functions in question are suppression of a gene 49 (Holliday resolvase) defect, UV-induced inactivation, and UV-induced mutagenesis. Out of this work emerged the hypothesis that EPR occurs in the immediate vicinity of a newly established recombinational intermediate. (The competing hypothesis, due to Radman and in vogue for nearly a decade, holds that a DNA-damage-induced protein leads to EPR by reducing polymerase fidelity; such a reduced fidelity has not been convincingly demonstrated in vitro despite many man-years of effort in several laboratories, two claims to the contrary being artefacts or irrelevancies.) More specifically, a DNA polymerase-blocking lesion (such as a dimerized pair of adjacent pyrimidines) must be bypassed ("bypass synthesis") by the polymerase for EPR to occur, and we believe that such bypass can occur more readily within the context of a D-loop than within an ordinary replication fork. This hypothesis provides the intellectual background for most of the objectives listed above.

Objective (5), however, reflects a different question. Until quite recently, defects in EPR, particularly those affecting primarily the mutagenic component of EPR (as contrasted, for instance, to error-free recombinational repair), produced only relatively small effects on cell or viral survival following treatment with diverse chemicals or radiations, especially compared with the large effects upon survival resulting from excision or recombinational repair. We had therefore long postulated that an important, perhaps the most important, adaptive advantage of EPR was the increase in genetic variance it engendered in response to environmental hostility. A competing hypothesis would be that nobody had yet examined the appropriate source of lethality, one that would be strongly repaired by EPR. Recently, however, the Eisenstadt group has reported that isopsoralen-mediated photodynamic inactivation proceeds many-fold faster in E. coli bearing a mutation specifically blocking the mutagenic component of EPR. We wish to see if the same holds for the T4 WXY system and, if so, if we can find other such agents. A happy technological byproduct of such a result would be a better agent than UV irradiation to use when screening for mutant alleles of the WXY system.

Existing technologies suffice for virtually all of our short-term objectives. The approach to the effect of ts mutants upon recombination is obvious. Tests for correlations between induced recombination and mutagenesis after UV irradiation are tricky but have been successful at least once in the past (in which proflavin, rather than UV, was the inducing agent). Tests for the effects upon EPR of other genes of DNA metabolism are conducted using conditional alleles of such genes under semi-permissive conditions, but must be conducted with great care to avoid the many pitfalls that have rendered most publications on the subject unacceptable. The screen for ts uvsW mutants cannot be conducted by traditional methods, since mutants in this gene have very modest effects on UV survival and do not suppress gene 49 defects; however, the two canonical uvsW mutants are both very sensitive to hydroxyurea, and this sensitivity should permit the isolation of ts mutants. The "uvsZ" mutant described in several papers and a thesis by Cupido is of interest because of his (thesis) report that it affects UV-induced mutagenesis. However, the technology used to measure mutagenesis was highly prone to artefact, the original isolate was lost, and our backcross to recover the mutant from a multiply mutant strain constructed by Cupido before loss of the canonical mutant led to segregants that probably included uvsZ but also included a totally unexpected strain, one that shows greater than wild-type resistance to UV. Despite strong selection, such a strain was never obtained in any of several laboratories in the past, and it will be of considerable interest to examine its properties in detail.

Our work on the mutant proteins of the WXY system will focus initially on ts alleles of uvsX, for which more background information is available about the wild-type protein from work in the Minagawa and Alberts groups. Three mutants will be used. All are quite strongly TS for suppression of a gene 49 defect. One is moderately TS for UV inactivation but strongly TS for UV mutagenesis, another is strongly TS for UV inactivation but only moderately TS for UV mutagenesis, and the third is TS for UV inactivation but shows little UV mutagenesis at any temperature. Protein purification is being conducted using conventional column procedures, and the proteins will be assayed for ssDNA-dependent ATPase, promotion of ssDNA annealing, promotion of D-loop initiation, and promotion of

branch migration. In addition, the uvsX proteins will be compared for affinity column binding to gene 32 protein and, perhaps, other T4 proteins (such as uvsY, dda and gene 43 proteins). Our hope is that differential behavior of the mutant proteins will indicate which of these traits are of particular relevance to mutagenesis.

B. Plans for Subsequent Year: Only about a third of the genetical studies outlined above can be completed within FY-84, and the remainder will extend through FY-85. We expect that the isolation of the mutant uvsX proteins will consume, at the least, the balance of FY-84, and that the examination of their properties will consume most of FY-85. If we have better-than-average luck with purification of the TS uvsX proteins, we will proceed to the purification of the TS uvsY proteins, since both proteins are required for EPR in T4.

C. Publications in Past 18 Months:

Sugino, A., Sakai, A., Wilson-Coleman, F., Arendes, J., and Kim, K.C.: In vitro reconstitution of yeast 2-μm plasmid DNA replication. In Cozzarelli, N.R. (Ed.): Mechanisms of DNA Replication and Recombination. New York, Alan R. Liss, 1983, pp. 527-552.

Drake, J.W.: Methyl methanesulfonate mutagenesis in bacteriophage T4. Genetics. 102: 639-651, 1982.

Drake, J.W., and Ripley, L.S.: The analysis of mutation in bacteriophage T4: delights, dilemmas, and disasters. In Mathews, K. et al (Ed.): Bacteriophage T4. Washington, Am. Soc. Microbiol., 1983, pp. 312-320.

Drake, J.W., Glickman, B.W., and Ripely, L.S.: Updating the theory of mutation: mutatis mutationum mutandis. Am. Scientist. 71: 621-630, 1983.

Sugino, A., and Drake, J.W.: Modulation of mutation rates in bacteriophage T4 by a base-pair change a dozen nucleotides removed. J. Mol. Biol. (in press).

Conkling, M.A., and Drake, J.W.: Isolation and characterization of conditional alleles of bacteriophage T4 genes uvsX and uvsY. Genetics. 107: (in press).

Conkling, M.A., and Drake, J.W.: Thermal rescue of UV-irradiated bacteriophage T4 and biphasic mode of expression of the WXY system. Genetics. 107: (in press).

Drake, J.W.: Viewing the complexities of mutagenesis from an evolutionary perspective. In de Serres, F.J. et al (Ed.): Genetic Consequences of Nucleotide Pool Imbalance. New York, Plenum Press, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60149-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular mechanisms of mutation: mutational specificity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. W. Glickman	Expert	LGM, NIEHS
	R. M. Schaaper	Visiting Fellow	LGM, NIEHS
Others:	R. L. Dunn	Biologist	LGM, NIEHS
	D. L. Halderman	Biologist	LGM, NIEHS
	R. A. Zakour	Senior Staff Fellow	LGM, NIEHS
	B. N. Danforth	Bio. Lab. Tech.	LGM, NIEHS
	R. G. Fowler	Guest Worker	LGM, NIEHS

COOPERATING UNITS *(if any)*
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Laboratory of Genetics

SECTION
Mutagenesis Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to make reliable estimates of the risks presented by environmental mutagens an intimate understanding of the mutagenic lesions, and the DNA repair processes that moderate them, is essential. The achievement of such detailed insights requires both quantitative and qualitative data on mutation at the molecular level. This project involves the production of mutational spectra over a range of doses in different repair backgrounds. The data produced will provide a basis for the identification of mutational lesions, and improve our understanding of the role of DNA repair in mutation avoidance and mutation fixation. Determination of mutational specificity is carried out in the lacI gene of E. coli. Using genetic tools, amber and ochre mutations are identified, mapped and characterized by suppression with known suppressors. Since the DNA sequence is known we then know the base substitution required to produce the mutation. In addition a rapid cloning and sequencing technique has been developed allowing us to determine mutational spectra at the DNA sequence level.

A. Research Project:

Problem: In order to make reliable estimates of the risks presented by environmental mutagens both qualitative and quantitative information on mutagenesis is required. Information on the properties of mutagenic lesions and the influence of dose and cellular repair capacities is required. Our approach to the identification of mutational lesions and an understanding of the DNA repair processes that moderate them involves the production of mutational spectra over a range of doses in different repair backgrounds. This data provides a basis for identifying mutational lesions and understanding the role of DNA repair in mutation avoidance and mutation fixation.

Objective: We wish to determine the DNA lesions responsible for mutation induced by several environmental agents and to investigate the role of DNA repair in the avoidance or fixation of mutation. As a model for this study we have concentrated ultra-violet light. Until recently UV was thought to exert its mutagenic effects through mutation targeted by pyrimidine dimers. However, adequate evidence now exists that another lesion, the (6-4) pyrimidine-pyrimidine photoproduct, might be of major importance. Studies on mutagenic specificity of UV, its kinetics and dependence upon DNA repair should enable a distinction to be made between the mutagenic potential of these two lesions. We have also asked questions concerning the source of spontaneous mutation and investigated the mechanism of mutation and the role of DNA repair following treatment with several environmental mutagens including ionizing radiation, alkylating agents. Metals and several base analogs have also been investigated. In addition, we have questioned the mechanism involved in mutagenesis induced by pool imbalances such as those observed during periods of thymine deprivation.

In our laboratory the determination of mutational specificity is most often carried out in the lacI gene of E. coli. In the past the production of mutational spectra has been carried out using genetical tools. LacI-mutants are selected and the amber and ochre mutations identified. These are then mapped and further characterized by an analysis of their suppression by known suppressors. The identification of the specific nonsense mutation induced and a knowledge of the DNA sequence reveals the base substitution required to produce the mutation. However, since this approach is restricted to the analysis of base substitutions capable of producing amber and ochre mutations de novo, frameshift, deletion and A:T → G:C events can not be monitored. We have thus set about developing a rapid method for the cloning and sequencing of lacI-mutations.

Accomplishments:

Spontaneous mutation: We have analyzed spontaneous mutagenesis in several repair deficient and mutator strains of E. coli. Among 1200 amber and ochre mutants in the lacI gene derived from the wild-type strain, all detectable base substitutions and all potential sites are represented. Three G:C → A:T hotspots have the sequence 5'-CCAGG-3' where the second C is methylated. At these sites spontaneous deamination yields thymine which unlike uracil is not removed by uracil-glycosylase and results in C → T transitions. Two hundred spontaneous mutants obtained in a glycosylase deficient strain (Ung^-) were all G:C → A:T

transitions, consistant with their predicted origin. The greatest fold-increases in mutation in Ung⁻ strains occur in A:T rich regions. Possibly deamination occurs during 'breathing' since spontaneous deamination occurs far more rapidly in single-stranded than fully hydrogen bonded DNA.

We have examined the recA-dependency of spontaneous mutation since most mutagens are recA-dependent. An analysis of 200 spontaneous mutants revealed no differences in the frequency or distribution of events. Similar results were obtained with a UmuC⁻ strain which like RecA⁻ strains are immutable by most mutagens.

The spontaneous spectrum was also determined in an excision repair defective strain (uvrB⁻). Spontaneous mutation was slightly elevated (1.7-fold) in the absence of excision repair, but no obvious alterations were noted. Sequence analysis is necessary to determine what events are responsible for the increase in mutation.

Most recently we have begun the determination of the spontaneous mutational spectrum in the wild-type strain. To date about 200 mutants have been sequenced, and base substitutions, frameshifts, deletion and insertion mutations identified. About 70% of the mutants occurred at a frameshift hotspot, nine were ISI insertions, 27 were deletions. An analysis of the deletions revealed that while many occurred between repeats, the majority could be explained as the result of the processing of secondary structures mediated by palindromes as we had predicted earlier (Glickman and Ripley 1984).

Mismatch repair and mutation: A significant component of cellular replication fidelity is due to a post-replication mismatch repair process (Glickman 1982 for a review). Strand discrimination relies upon adenine methylation under the control of the dam gene (Glickman et al. 1978). Dam⁻ strains are powerful mutators showing enhancement of transition and frameshift mutation (Glickman 1979). Other mismatch repair mutants have been isolated (Glickman and Radman 1980, Glickman 1982). These mutators which include mutH, mutL, mutS, uvrE (uvrD) and recL all demonstrate increased transitions and frameshifts. However, each displays a unique spectrum, an indication that the proteins encoded by these genes interact. In addition, we have studied induced mutation in these strains. In particular, conditions which challenge the fidelity of DNA replication might reveal a role for mismatch repair.

1) Alkylation: We have studied the role of DNA repair on alkylation-induced mutagenesis (e.g. Todd et al 1981) including the role of mismatch repair (Glickman 1982, Glickman, Dunn and Halderman, in preparation, Marla Babcock-Harms (thesis 1983) and Babcock-Harms and Glickman, in preparation). MutH, L and S mutations confer increased resistance to dam⁻ and wild-type. strains to killing by MNNG. They also enhance MNNG-induced mutagenesis. However, the induced spectra are similar to those recovered in the wild-type strain. Mismatch repair thus reduces MNNG mutagenesis but attempts at repair may result in lethality. 2) Thymine deprivation: Thymine deprivation leads to cell death and mutation. We have analyzed the specificity of thymineless mutagenesis in wild-type and Ung⁻ strains (Kunz and Glickman 1984) and mismatch repair mutants. The mutH, L and S alleles confer modest resistance to thymineless death and enhance mutagenesis (Glickman and Skrzynski, in preparation).

This is consistent with a role for mismatch repair as a post-replicational process and suggests that its action, while reducing mutation, can also result in lethality. 3) Ultraviolet light: Models of error-prone repair usually depend upon reduced replicational fidelity for the bypass of bulky lesions (see Walker 1984 for a recent review). The reduction in replicational fidelity is manifest by such phenomena as e.g., Weigle or indirect mutagenesis or untargeted mutagenesis (see Kunz and Glickman 1984). Since UV mutagenesis requires the induction of error-prone repair it might not be limited to sites of DNA damage but could also result from lowered replicational fidelity and hence be untargeted. In E. coli most UV-induced mutation appears to be targeted (Coulondre and Miller 1977, Todd and Glickman 1982, Glickman 1983). In bacteriophage M13 however, untargeted mutations are recovered (Brandenburger et al 1981, Schaaper and Glickman 1982). One explanation for this difference might be that M13 is not subject to mismatch repair. We therefore determined the specificity of UV in the absence of mismatch repair and found considerable levels of untargeted mutagenesis in a mutH strain! Thus, mismatch repair can function to avoid errors potentiated by error-prone repair. In addition, mismatch repair mutants confer some degree of UV-resistance (Glickman 1982).

MUTATIONAL SPECIFICITY AND ULTRAVIOLET LIGHT

The power of mutational specificity to resolve the nature of premutagenic lesions and the effects of DNA repair is vividly demonstrated by the study of UV-induced mutagenesis. Historically, pyrimidine dimers have been assumed to be the premutational lesion. More recently however, another UV photoproduct, Py-C or the (6-4) pyrimidine-pyrimidine photolesion has been suggested to be responsible for UV-induced mutagenesis (Brash and Haseltine 1982). We thus undertook to examine more closely the specificity of UV-induced mutagenesis. Experiments with photoreactivation, kinetics of mutation induction at individual sites in the lacI gene revealed distinct differences suggesting that more than one type of lesion might play a role in UV-induced mutagenesis. Experiments with the transfer of irradiated F's (Kunz and Glickman 1984) demonstrated quite convincingly that pyrimidine dimers are capable of targeting mutation. An experiment described below demonstrates that (6-4) lesions may also target mutation.

Thymine residues are poor substrates for (6-4) photoproduct formation. Similarly these lesions form poorly at sites containing 5-methylcytosine whereas dimer formation appears to be unaffected. Should the (6-4) lesion be of major importance in UV mutagenesis then 5MeC sites might be expected to be poor responders to UV light. In the lacI gene, there are three such sites. In the wild-type strain these sites account for less than 4% of all mutants recovered at 11 sites where the (6-4) lesion or dimer can be induced and lead to an amber mutation by a G:C → A:T transition. In contrast, the analysis of more than 900 UV-induced amber mutants in a Dcm-strain (which thus lacks 5MeC in its DNA) revealed 19% of all amber mutations occur at these sites. This observation strongly suggests that the additional mutations were targeted by (6-4) photoproducts.

MUTATIONAL SPECIFICITY OF IONIZING RADIATION

We have examined the mutational specificity of ionizing radiation in the lacI system. Our initial experiments involved 60Ca gamma-rays (Glickman et al 1980)

and demonstrated that 1) all possible base substitution were induced and 2) most sites appeared to respond similarly. No obvious relationship was noted between the spontaneous spectrum and that obtained following gamma-irradiation. Data obtained suggested a dose-dependent effect and spectra were produced over a range of doses. Analysis of these revealed that at low doses (e.g. the doubling dose) transitions predominated while at high doses transitions and transversions were induced with equal frequency (Glickman 1982b, Glickman, 1984). Our work on the mutagenic effects of tritium decay is outlined elsewhere in this proposal.

MUTATIONAL SPECIFICITY OF METALS

The ubiquitous nature of metals in the environment and the causal relationship between certain metals and cancer prompted us to study the mutational potential and specificity of Be, Mn and Cr (Zakour and Glickman 1984). These studies proved interesting as we were able to show that different metals must operate via different mechanisms as each metal tested produced a unique spectrum. In addition, the effectiveness of the lacI system to detect mutation at very low levels was demonstrated.

OTHER TREATMENTS

We are presently determining the mutational specificity of spontaneous events in a PolA, MutD (in collaboration with Dr. R. Fowler presently on sabbatical from San Jose State University) and Dut-strains, both by the analysis of nonsense mutations and DNA sequencing. Each of these strains show a high spontaneous mutation rate and a unique mutational spectrum. Analysis of these data should provide some insights into the source of the mutations.

MUTATIONAL SPECTRA BY DNA SEQUENCING: A RAPID METHOD FOR THE SEQUENCING OF *lacI* MUTANTS

Detailed analysis of mutational specificity requires information at the DNA sequence level, which requires a rapid and efficient method for the cloning and sequencing selected mutants. The original method used for the sequencing of *lacI* mutations made use of the plasmid vector pMC1 which carries the lacI gene (e.g. Farabaugh et al 1978). In order to be sequenced, the mutations were transferred from the F' to the plasmid by homogenization (in vivo recombination). The main disadvantage of this system is that most of the *lacI* mutations recovered on pMC1 de novo in origin.

Our approach to the cloning and sequencing of lacI mutants circumvents these difficulties by requiring concommittant events. We have constructed an M13 derivative which is $lacI^+$ but carries a mutation in the *lacZ* gene, enabling us to select for $lacI^-$ recombinants that have simultaneously become $lacZ^+$. Control experiments show that this approach effectively eliminates the sequencing of de novo lacI mutants.

The experimental procedure involves three steps:

1) The crossing of the $lacI^-$ mutation onto the M13 derivative by the selection of $lacI^-lacZ^+$() recombinants as outlined in Figure 1. The M13 $lacI^+lacZ^-$

derivative is grown on the lacI⁻ bacteria and the resulting bacteriophage are plated on media containing X-gal but no inducer of the lac operon. Blue plaques are selected (They are lacI⁻lacZ) and plaque purified.

2) The genetic localization of the lacI⁻ mutant is carried out by deletion mapping positioning the mutation to within +/- 75 base pairs.

3) The DNA sequence is determined by the dideoxy method using synthetic primers conveniently spaced every 150 base pairs throughout the gene.

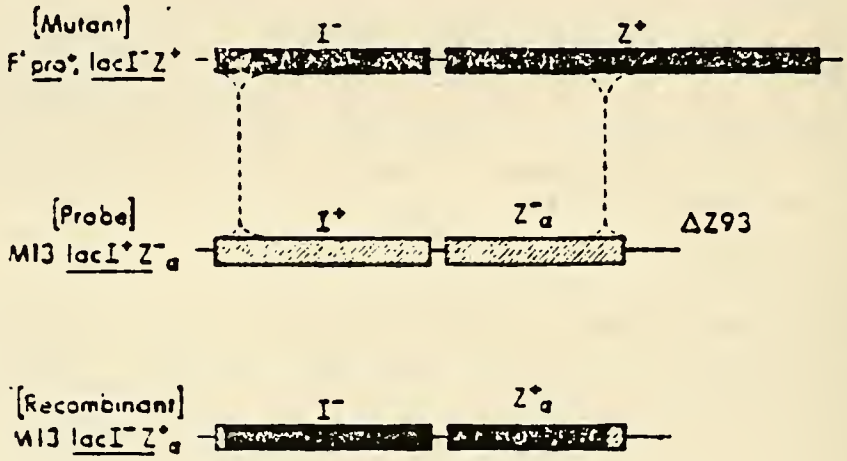


Figure 1.

This procedure is rapid. Mutants can be transferred to M13 and be sequenced within a week. Spontaneous, i.e., de novo mutations on the M13 are effectively avoided (1 per 5000 recombinants) since those which arise spontaneously would also have to become lacZ⁺ as well. At present we estimate that between 120 and 160 mutants can be sequenced per person per month.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60150-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Induction by Alkylation Treatments in E. coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. R. Volkert	Senior Staff Fellow	LGM, NIEHS
Others:	D. C. Nguyen	Chemist	LGM, NIEHS
	K. C. Beard	Q	LGM, NIEHS

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

When E. coli cells are treated with sublethal levels of alkylating agents, they become more resistant to the mutagenic and lethal effects of subsequent high level treatments with alkylating agents. This increased resistance has been called the adaptive response and is the result of the induction of increased capacity to repair alkylation lesions in DNA. We have initiated a genetic study designed to learn what genes are induced by alkylation treatments, how they are regulated and what the functions of their products are. To date we have identified five genes or operons that are specifically induced by alkylation treatments by constructing fusions of the lac operon to promoters of genes induced by alkylation treatments. One of these alkylation inducible (aid) genes, or operons, (aidA) codes for the alkA gene product which is a glycosylase that removes methylated bases from DNA. aidD appears to be a fusion to the ada operon, which codes for two gene products, at least one of which is a regulatory protein controlling the adaptive response. The other three loci represent new genes involved in this process. Our studies show that treatments with levels of alkylating agents that elicit the adaptive response are suboptimal, and induce only a subset of the alkylation inducible genes. At optimal concentrations of alkylating agents, additional genes are induced. In addition, only four of the five loci are regulated by the ada gene. The fifth locus, aidC, is not only ada independent but is induced only by some methylating agents and not others. Thus the induction of genes by alkylation treatments is more complex than was originally revealed by studies of the adaptive response. We are now using these strains to learn how these aid genes are regulated and how their products function at the molecular level.

A. Research Project:

Problem: When E. coli cells are treated with low levels of alkylating agents they become more resistant to the lethal and mutagenic effects of subsequent high level challenge treatments with alkylating agents. This induced resistance has been termed the adaptive response, and is due to the induction of at least two gene products that act to repair specific alkylated nucleotides and phosphotriesters. The two major types of activities that are increased during adaptation are glycosylase activities and methyltransferase activities. The glycosylase activities, which are encoded by the alkA gene, act to cleave the glycosylic bond between the base and the sugar of the DNA backbone, removing in this fashion, several alkylated bases (N^3-methyladenine, N^3-methylguanine, N^7-methylguanine, O^2-methylcytosine, and O^2-methylthymine). Methyltransferases act to remove the alkyl groups from several alkylated bases in DNA, restoring the original base to its unmethylated state. Methyltransferases act specifically to repair O^6methylguanine, and O^4-methylthymine. In addition, a methyltransferase activity was recently described that removes the methyl group from methylated phosphotriesters in the sugar-phosphate backbone of the DNA.

Although biochemical studies of the adaptive response have resulted in the characterization of several induced enzymatic activities, little is known about the genes coding for these activities. To date only the alkA gene and a regulatory gene called ada have been identified. Moreover, the full scope of the adaptive response has not been adequately studied as it is not known how many other genes are induced in response to alkylation treatment, nor is it understood how such genes are regulated.

Experimental Approach and Accomplishments: Our approach to the genetic aspects of the adaptive response has been to construct random operon fusions of the lac operon to E. coli genes using the phage Mu-dI(Ap^Rlac). This phage contains the lac operon but is deleted such that the β-galactosidase gene (lacZ) has no promoter. β-galactosidase activity can be expressed only when this phage has been inserted into a gene in the proper orientation such that transcription begins at the promoter of the target gene and proceeds into the lacZ gene of the phage. In addition to allowing β-galactosidase activity to be expressed, this type of insertion places the lacZ under whatever regulatory control is exerted on the external promoter. Thus, by selecting for fusion containing strains that express β-galactosidase only after treatment with alkylating agents, it is possible to select for fusions to genes that are normally induced in response to alkylation treatment. In addition to providing a way to select for fusions to alkylation inducible genes regardless of gene function, the resultant fusion provides a convenient and useful tool with which to measure gene expression simply by measuring β-galactosidase activity. Mutant phenotypes often result whenever the sequence of the structural portion of the target gene is interrupted by the phage genome. Thus, the function of the target gene can often be deduced from phenotypic characterization of the fusion mutant.

To date we have isolated a total of 9 lac fusion strains representing at least 5 different genetic loci that are induced specifically by alkylation treatments. Included among these 5 are fusions to the alkA promoter and the ada promoter. The isolation of these two mutants has allowed us to quantify the induction of

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 60151-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of the recF Gene in DNA Repair

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. R. Volkert	Sen. Staff Fellow	LGM, NIEHS
Others:	M. A. Hartke	Q	LGM, NIEHS

COOPERATING UNITS *(if any)*
Alvin J. Clark and Linda J. Margossian
Department of Molecular Biology, Univ. of California
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LAB/BRANCH
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INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The recF gene is required for two basic genetic processes, genetic recombination and DNA repair. The major question about recF function centers on whether recF functions in a regulatory capacity controlling these processes or whether it has an enzymatic function needed to carry out these reactions. Our studies of suppression of recF by several recA mutations suggests that the recF gene product functions primarily by modulating recA activity. It appears that two recF dependent changes in recA activity normally occur in response to DNA damage. The first change results in activation of the recA dependent proteolytic activity required for induction of the SOS pathways of DNA repair. The second change allows recA to carry out the RecF pathway of recombination and recombinational DNA repair. These two changes in recA activity are separable by mutation since the srfA mutation of the recA gene results in restoration of only RecF recombination without concomitant restoration of SOS induction in recF mutant strains.

A. Research Project

Problem: The recF gene of E. coli is required for a UV inducible pathway of recombinational DNA repair. This pathway of recombinational repair can also function to carry out general genetic recombination in the appropriate genetic background. In addition, recF is also required for expression of the inducible SOS pathways of DNA repair after UV treatment. The induction of the SOS response is a two step process. The first step is the activation of the RecA protein, the second is the cleavage of LexA and other repressors of SOS genes. This proteolytic cleavage results from the interaction of the activated RecA protein with the repressors and causes the induction of the genes they control.

The understanding of how these two gene products function in recombination and repair will aid our understanding of the cellular responses to DNA damage. This work will provide information about how such repair and recovery processes are regulated and how they function at the molecular level.

Experimental Approach and Accomplishments: Our first approach to the study of the role of recF was to introduce the recA441 mutation into a recF mutant strain. This recA mutant allele produces an altered RecA protein which can be activated to cause expression of the SOS response in the absence of DNA damage, simply by incubating the cells at 42°C. Under these incubation conditions, recA441 suppresses many of the recF defects and circumvents the need for recF in SOS induction. This suggests that the recF gene may function to modulate recA activity, catalyzing the conversion of recA from its inactive to its activated form after UV damage. Experiments designed to determine if the RecF pathway of recombination was also restored under these conditions were equivocal.

In order to learn more about the interaction of recF with recA, we have isolated suppressor mutants that restore UV resistance to recF mutant strains. One class of recF suppressors which we call srfA map at recA and restore the recF pathway of recombination and recombinational repair.

Since srfA mutations restore only RecF recombination to recF mutants and do not restore SOS induction capabilities, the mechanism of suppression of recF by srfA differs from that of recA441. However, both types of suppression are a result of alterations in recA activities. Since both types of recA changes restore different components of recF dependent process, we have proposed that in wild-type cells recF normally functions to modulate recA activities, and that two changes in recA activity occur after UV damage. The first change is its activation to its proteolytically active form resulting in SOS induction. The second change allows recA to carry out the RecF type of recombination. Although these changes normally occur simultaneously after DNA damage they are separable by mutation since srfA mutations restore only the recombinational activity to recF mutants.

Future Directions: Our current efforts on this topic are designed to quantify the effects of srfA mutations on SOS induction in recF mutant strains, to determine which aspects of the recF phenotype are restored by srfA, and to determine where in recA the srfA mutations lie.

Publication:

Volkert, M.R. and Hartke, M. A.: Suppression of Escherichia coli recF mutations by recA-linked srfA mutations. J. Bacteriol. 157: 498-506, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61005-05 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis and function of RNA Polymerase II in Drosophila melanogaster

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. A. Voelker	Research Geneticist	LG, NIEHS
Others:	L. L. Searles	Staff Fellow	LG, NIEHS
	S. S. Huang	Bio. Lab. Tech.	LG, NIEHS
	G. B. Wisely	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

Dr. Arno Greenleaf, Department of Biochemistry
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LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study was initiated to genetically analyze the biosynthesis and function of the components of the RNA polymerase II transcription complex in Drosophila melanogaster. RNA polymerase II is a heteromultimer consisting of approximately ten different subunits, each of which is presumably specified by a different locus. The number of associated transcription factors (which are not structurally a part of RNA polymerase II) is unknown, but evidence for their existence has been found in other systems. To date only the genetic locus which specified α-amanitin-resistance to RNA polymerase II has been identified. That locus has not been cloned as recombinant DNA molecules and was found to encode the 215,000 dalton subunit. The genetic control of the biosynthesis of that subunit is being analyzed at the molecular level by analyzing a number of revertants of the P-element induced mutant that was used to clone the DNA sequences of the region.

PROJECT DESCRIPTION

PROBLEM: RNA polmerase II, which is a heteromultimer consisting of about ten different subunits, is the enzyme complex that functions in the transcription of messenger RNAs in eukaryotic organisms. This study was initiated to genetically analyze the genes involved in the biosynthesis and functions of this enzyme complex.

OBJECTIVES: The objectives are to identify the genes encoding the various subunits of the complex and characterize them at the molecular level. Understanding the genetic control of the coordinate biosynthesis of the complex is a long-range goal.

METHODS EMPLOYED: The basic approach of this study is to utilize genetic techniques of analysis to identify mutants of RNA polymerase II that can be subsequently analyzed at the biochemical and molecular levels. The one locus identified was discovered by screening for α-amanitin-resistance. The locus was mapped by standard recombinational and deletion mapping techniques. It was further characterized by induction and genetic/molecular characterization of additional mutants at the locus.

MAJOR FINDINGS AND PROPOSED COURSE: A locus that confers α-amanitin resistance to RNA polymerase II and encodes the 215,000 dalton subunit has been identified. The locus is lethal-mutable. Different alleles at the locus affect male fertility and act as enhancers of alleles at the other loci. The C4 locus has been molecularly cloned by identifying and recovering mutants at the locus that were caused by hybrid-dysgenesis-induced insertion of the P-element transposon. One of these P-element-induced mutants has been found to be genetically very unstable in dysgenic flies. It reverts with frequencies of 2.5 to 6.5 percent by excision of the P-element, with many of the revertants resulting from imprecise excision. The consequences of these imprecise excisions on function were analyzed. The completion of this genetic/molecular analysis completes the involvement in this project. Further work on the problem will continue in the laboratory of Dr. Arno Greenleaf in whose laboratory the project was initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: RNA polymerase II is an important enzyme in eukaryotic gene regulation and development. The details of the role of this enzyme are very poorly understood, and the prospects of a comprehensive genetic and biochemical analysis of such a complex enzyme with mammals are very bleak. Therefore, we have chosen to approach the problem with Drosophila, a well-defined eukaryotic genetic system which allows a powerful combination of genetics and biochemistry. Moreover, it appears that the structure and function of Drosophila RNA polymerase II is very similar to that in humans and other mammals; thus knowledge obtained in Drosophila can probably be transferred to and utilized in the human situation with relatively little modification.

Before we can understand the risks of environmental mutagens and carcinogens, we must know how they affect the fundamental processes of cell growth and development. The effort of this study is to determine the role of RNA polymerase II in

normal cell function. When that knowledge is available we can begin to assess how the various environmental insults impinge on normal cellular function.

PUBLICATIONS

Voelker, R.A., Greenleaf, A.L., Gyurkorics, H., Wisely, G.B., Huang, S.-M., and Searles, L.L.: Frequent imprecise excision among reversions of a P element-caused lethal mutation in Drosophila. Genetics 107: 279-294, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61011-05 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Organization and Regulation of Gene Function in D. melanogaster

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	B. H. Judd	Research Geneticist	LG, NIEHS
Others:	Margaret W. Shen	Biologist	LG, NIEHS
	Patricia S. Davis	Chemist	LG, NIEHS
	Deborah A. Adams	Guest Worker	UNC, Chapel Hill

COOPERATING UNITS *(if any)*

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Laboratory of Genetics

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This study involves the genetical, cytological and molecular characterization of the white locus of Drosophila melanogaster. Comparisons of the normal gene structure with those of mutants that perturb the regulation of the locus are being made in an effort to understand the nature of the mutational changes and how they effect the function of the locus. The mutations currently under investigation result from the insertion/deletion of transposable elements near the 5' end of the gene.

Some of the mutants exhibit a mosaic of normal and mutant patches in the eye that reflect a clonal expression of the white gene while others are mosaic and show a nonclonal, nonautonomous expression. The two types of mutants are related to each other by descent and the objective is to determine the nature of the mutational changes and try to relate them to the way that regulation of the locus is modified.

PROJECT DESCRIPTION

PROBLEM: This analysis of the white locus of Drosophila melanogaster addresses questions about the organization, function and regulation of eukaryotic genes. We want to understand the mechanism through which genes respond in developmentally regulated patterns to produce the multiple cell and tissue types of complex organisms. The white locus provides a model system through its many mutant forms and the interactions it has with other loci.

OBJECTIVES: The objectives of this study of mutant alleles that perturb the regulation of the locus are to provide information about the nature of the regulatory elements of the gene and to learn about the nature and mechanisms of gene mutation.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The analysis is by genetic, cytological and molecular characterization of specific genes in D. melanogaster. Genetic analysis involves recombination, complementation tests and induction of mutations by mutagens or through hybrid dysgenesis. Cytological preparations provide information about chromosome aberrations and the in situ hybridizations of labeled DNA probes give information that ties together the genetical and molecular information concerning gene structure. Molecular techniques include the cloning of specific gene sequences in recombinant DNA molecules, the restriction enzyme digestion of DNA, Southern blot analysis, nick translation of specific DNA probes, and nucleotide sequencing.

The study focuses on the mutation w^{zm} which produces a mosaic pattern of eye pigmentation. This mutation is associated with the insertion of a transposable sequence of DNA near the 5' end of the transcribed portion of the gene and it exhibits a moderate instability. Mutant derivatives of w^{zm} show striking modifications of gene regulation, with some exhibiting mosaic patterns that are clonal and autonomous ($w^{z\ell}$), others completely lack any pigmentation (w^{z}), while the most unusual group shows a nonclonal, nonautonomous mosaic of pigment deposition (w^{zn}). It has been determined that the w^{z} mutation is the result of a second insertion of nonwhite locus DNA about 3.5kb from the site of the w^{zm} insertion. The nonclonal, nonautonomous type such as w^{zn} has lost most of both the insertion sequences in w^{z}; in addition the entire white locus as well as some adjacent genes have been transposed to the left arm of chromosome 3. We plan a detailed analysis of the gene regions into which the transposons are inserted and a comparison of different mutant forms to determine how they affect the normal regulation of the locus. Of particular interest is a comparison of the mutants showing the clonal expression of the gene compared to those giving nonclonal action. In both there seems to be improper gene activation but this happens in some cells only. In the clonal form those cells that have proper activation, pass the activated state on through several mitotic divisions of daughter cells. In the nonclonal mutants this mitotic memory is not functioning or possibly the locus is not activated in the proper tissues, resulting in nonautonomy.

Another aspect of white locus structure being investigated is the molecular basis for intralocus asymmetrical exchanges that result in small duplication and

deficiency recombinant chromosomes. Preliminary data implicate a repeated transposable DNA sequence, B104, that is associated with the w^{bf} mutation. This element apparently is located at a different place in or near the white locus of the w^{a} chromosome. Pairing and exchange between the B104 sequences in the two chromosomes creates the duplication and deficiency products. These products are useful in characterizing regional differentiation within the locus and for studying the details of the mechanisms involved in crossing over.

A third approach to understanding white locus function is through investigation of mutants that show a chromosome pairing dependent expression. The mutant w^{sp} produces a mosaic of pigmented cells on a very lightly colored background. It complements with most other white alleles to give a uniformly pigmented eye if the structure of the homologous X chromosomes is standard. If, however, a rearrangement is introduced into one of the chromosomes such that somatic pairing is upset, this complementation is abolished. Deborah Adams is examining this phenomenon to determine how the two alleles communicate with each other and how that communication is perturbed when homologs cannot pair.

RECENT ACCOMPLISHMENT AND SIGNIFICANCE: The entire element inserted into the proximal portion of the white locus that causes the w^{zm} mutation has been cloned into a lambda phage ($\lambda w^{zm}1$-17). It is about 6.3kb in length and we have done a partial restriction map of the element. Using ^{3}H-labeled copies of this clone hybridized in situ to polytene chromosomes, we have determined that the element is found at about ten sites interspersed in the chromosomes of the Oregon-R wild type strain. Copies are found at the white locus in w^{zm} and its X chromosome mutant derivatives and in 3L at 61D in the w^{zh} derivatives. A copy of $\lambda w^{zm}1$-17 was sent to Dr. Robert Levis at Berkeley. By nucleotide sequencing, he has located the insertion site of the transposable element in an intron near the 5' end of the white gene about 16bp from the splice site.

Extensive restriction digest and Southern blot analysis of w^{bf}, w^{a} and the unusual recombinant chromosomes shows at least three different types of recombinant events can occur in the white locus region in w^{bf}/w^{a} heterozygotes. The transposable elements copia and B104 are apparently involved in influencing the abnormal pairing and exchange.

The analysis of the pairing dependent gene expression is in a very preliminary stage. Mutants and new chromosomal rearrangements are being sought that modify the expression of the locus and the mRNAs generated by several genotypes (complementing and noncomplementing) are being analyzed.

Future plans are to characterize fully these several categories of mutants that upset gene expression and to try to understand how genes are regulated in the development of specific cell types in multicellular organisms.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61018-04 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

DNA Sequence Variation in the Alcohol Dehydrogenase Gene Region of Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles H. Langley	Research Geneticist	LG, NIEHS
	Charles F. Aquadro	Staff Fellow	LG, NIEHS
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	Brian Golding	Guest Worker	LG, NIEHS
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Variation in the DNA restriction map in the Adh region (alcohol dehydrogenase locus) of chromosome II of Drosophila melanogaster from natural populations was examined. On average, 1 out of 50 nucleotides was observed polymorphic in the 12 kilobase region examined. In addition, we found insertions and deletions to be common, particularly in regions flanking the Adh transcript. All insertions of over 200 nucleotides share sequence homology with known transposable elements. The frequency distribution within Drosophila melanogaster and among related species suggests that such variants are deleterious mutants. Comparisons of Adh gene activity among inserted and noninserted sequences support this view for some variants. The effect of other flanking sequence variants may be on other adjacent genes and/or general chromatin structure. Evidence is found for preferential insertion of transposable elements into sequences implicated as being important in gene regulation (identified by their DNAse I hypersensitivity in intact chromatin). Two levels of Adh activity (high and low) commonly segregating in natural populations appear due to one or more nucleotide substitutions within the transcript that in some way alter standing levels of Adh mRNA (possibly by affecting mRNA stability or processing). These activity level substitutions appear to be distinct from, but in strong nonrandom association with, the two commonly segregating alleles of the protein (which differ by a single amino acid). It is clear that the target for mutations of significant effect is substantially larger than the coding sequence for the gene product.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: Our knowledge of the quantity and significance of genetic variation is largely limited to transcribed regions of the genome. However, these regions represent only an extremely small portion of the eukaryotic genome. Recent technical advances in molecular biology and DNA technology have made it possible to examine in detail the previously inaccessible noncoding genetic material. Studies have revealed a remarkable diversity of variation can exist in these noncoding regions, yet the significance of much of it remains unknown. Also, we know little of the role these sequences play in proper gene expression. What is the target for mutations of significant effect?

OBJECTIVES: We have focused on an intensively studied genetic locus in Drosophila melanogaster, that encoding the enzyme alcohol dehydrogenase (Adh). Our objectives have been to discover 1) What levels of DNA sequence variation normally occur in natural populations (i.e., insertions, deletions, nucleotide substitutions, rearrangements, transposable elements, etc.); 2) Where, relative to structural genes or other regions of defined function, these alterations occur; 3) What effects, if any, these alterations have on the expression of the genes in these areas of the genome?

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: We have chosen to study D. melanogaster from natural populations for several reasons. The genome size of Drosophila is more than an order of magnitude smaller than that of mammals, making the studies significantly more feasible technically. There also exists a tremendous wealth of genetic and molecular data for the Drosophila genome (and the Adh gene in particular) on which to draw as a resource. In addition, the documentation of, for example, the effect of insertions of transposable elements on the expression of adjacent genes is extremely difficult without detailed genetic mapping studies of the gene activity effects. Such studies are infinitely more feasible in Drosophila than in mammals. The study of naturally occurring DNA sequence variation is also central to the mission of the Institute because it provides information on the background levels of variation, information vital to the assessment of the significance of mutagen exposure. The results also contribute significantly to determining the "target" for mutagenesis and the types of variation that can and cannot be tolerated.

Forty-nine genetically isolated second chromosomes of D. melanogaster were reared and nuclear DNA isolated. Restriction endonuclease site maps were constructed for a 12 kilobase region including the Adh structural gene. These maps were then compared for evidence of nucleotide substitutions and insertion/deletion variation. Insertions and deletions were cloned and characterized by restriction mapping, sequencing and comparison of sequence homology with previously described transposable elements. Adh gene expression was measured in adults by assaying ADH enzymatic activity. Some lines were also examined at the larval stage and analyzed for altered levels of Adh-specific messenger RNA. An additional 60 lines of D. melanogaster representing additional natural populations and variants were also examined for restriction map variation in the Adh region.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: We have mapped and scored 31 restriction sites, eight of which were polymorphic. From the frequency of site variation in these populations we estimate that 1 in 50 nucleotides vary and that between any two randomly chosen chromosomes, 1 in 200 nucleotide sites will differ. All lines have also been scored for their ADH allozyme (Fast vs. Slow) by starch gel electrophoresis. This difference in mobility is due to a Thr vs. a Lys at amino acid position 192 and reflects a single nucleotide substitution.

More remarkable than the extent of nucleotide substitution variation is the diversity of sequence length variation. Eighty percent of the lines have at least one insertion or deletion relative to the most common restriction map. These insertions/deletions range in size from 27 to over 9000 bp in length. This length variation is distributed over most of the 12 kb region with some notable exceptions. Clustering of length variation is apparent approximately 1.5 kb 3' to Adh. Four sizes of inserts, ranging from 340 bp to over 9 kb, were observed in a 340 bp region in eight of the 49 lines (one insert, "b", occurs in five different lines). No insertions/deletions were observed in Adh coding sequence or in the immediate 3' untranscribed region. Three small (27 to 38 bp) insertions/deletions were, however, located in sequence corresponding to the 5' untranslated portion of the adult Adh transcript.

In an effort to determine the origins of and mechanisms resulting in the sequence length variation observed, we have cloned and characterized each insert. Length variation fell into two classes: unique sequence events (DNA involved not present elsewhere in the genome) or repeated sequence events due to the presence of transposable elements. Unique sequence length variation was of two types. Two insertions, present in 30% of the lines, were estimated to be 31 and 34 bp in length. Seven deletions of unique sequence were also observed in 61% of the lines, and ranged in size from 21 to 200 bp.

Seven sizes of inserts, observed in 11 lines, were found to be repeated throughout the genome. A five kb insert, occurring approximately 0.4 kb 5' to the beginning of the adult Adh transcript in one line (RI42), appears to represent a complete copia element while a 4.8 kb insert 3' to Adh in another line appears to be homologous to an F element. Interestingly, all 11 inserts (4 sizes) in the approximately 230 bp EcoRI/SalI fragment 1.5 kb 3' to Adh share sequence homology with a relatively uncharacterized repetitive sequence, termed 2161. Our sequencing shows 2161 to be a member of the F-family of elements by virtue of its structure, in particular, a poly-A end and lack of direct or inverted repeats. The three smaller inserts at this site (340, 400 and 700 bp) have homology to 2161 alone, and represent abreviated and/or defective copies of a complete element. However, the fourth insert in this EcoRI/SalI fragment (an insert of over 9 kb) shows homology to 2161 at both ends, whereas the central 9 kb represents an apparently complete copy of the element B104. The remaining 3' repetitive insert, "n", appears to represent a 400 bp piece of a B104 element.

One approach to assessing the significance of this enormous amount of sequence variation, most of which occurs in nontranscribed regions, is to ask whether it is associated with altered expression of adjacent genes. A readily assayed index of gene expression at Adh is enzyme activity. While ADH activities are

spread over a five-fold range, two clusters of lines are apparent; termed here high and low activity. Of particular interest is the nearly complete association between the Fast allozyme and high activity, and the Slow with low activity. We were interested in whether the high/low difference was due to the Thr to Lys replacement, which underlies the allozyme difference or perhaps was due to a flanking sequence variant. Our results suggest the answer to both questions is no.

Anderson and McDonald (1983) have demonstrated that the difference in activity per fly between the two allozymes is due to a difference in rate of protein synthesis, not in rate of degredation. This is in agreement with the observation that the higher activity of fast lines is correlated with higher levels of ADH protein and ADH-specific mRNA suggesting the protein level differences are primarily attributable to differences in ADH message levels (Anderson and McDonald, 1983, and our work). It might be argued that the A to C substitution responsible for the Lys to Thr replacement is also the source of the variation in mRNA levels. Our restriction map data suggest that this is probably not the case. There are three obvious activity outliers among the lines we have studied; that is allozymes associated with the "wrong" activities. Heat and urea denaturation studies and comparison of activity ratios for different alcohol substrates suggest these lines do not have new amino acid substitutions.

We found strong nonrandom associations among variants in the 12 kilobase region, including the allozyme polymorphism and ADH activity. In addition, activity levels show a pattern generally consistent with the phylogeny constructed for the 49 restriction maps and the distribution of the fast/slow polymorphism. There was a clearly defined split in the phylogeny between slow and fast haplotyes, consistent with Kreitman's (1983) DNA sequences from the region immediately surrounding the Adh transcribed region. The restriction map for one of the lines with the "wrong" activity for its allozyme (NC16, Fast but low activity) appears to be recombinant in the region just 5' to the Adh allozyme (Fast Adh and 3' region but a 5' region typical of a Slow with low activity). Another line could be considered either a 5' recombinant or an evolutionary intermediate in the evolution of fast from slow. Importantly, recombinants 3' to Adh or possible recombinants in the extreme 5' end of the 12 kb region do not show shifts in ADH activity.

The activity differences for these lines are tightly linked to the allozyme polymorphism (in fact, no recombinants have been observed in the experiments carried out to date) and the effect is cis-acting. In addition, the "switch" from high to low activity in NC16 is associated with a switch from high to low level of Adh mRNA and is seen at both the adult and larval stages. These results are consistent with the presence of a polymorphic "regulatory" type site within several kb 5' of the Adh allozyme site, one allele at the site leading to approximately twice the level of Adh expression as the other (high and low, respectively). The difference (or differences) between these two "regulatory alleles" is apparently due to nucleotide substitution and not insertion/deletion as none of the latter are observed strongly correlated with activity.

In order to test our hypothesis and attempt to refine more closely the precise location of the putative "regulatory" site, we obtained nine D. melanogaster

lines from natural populations for which Dr. M. Kreitman had determined the DNA sequence for a 2.5 kilobase region surrounding and including the Adh gene. Our restriction mapping of these lines extended DNA sequence markers to the full 12 kb region and indicated two apparent natural recombinants (or convertants) on the 5' side of the Lys to Thr amino acid replacement. Subsequent determination of ADH activity, however, showed no "switch" in activity relative to the allozyme. Thus, these data (together with our other data) suggest that the nucleoti responsible for the major between allozyme difference in Adh activity and messenger RNA levels is a third codon substitution contained within the third exon of the ADH protein. The effect on this substitution of standing levels of Adh messenger RNA could result from differences in the rate of transcription, the rate of processing and/or the stability of the transcript.

As with the between-allozyme component of activity variation, there is a tendency within an allozyme class for similarity in ADH activity level to be correlated with propinquity of descent of the lines based on the Adh region. One might propose the transposable element insertions or other insertion/deletions flanking Adh may be the source of some of this activity variation. However, no clear pattern emerged from an examination of the different types of events as classes. For example, transposable elements are present in the majority of the lines having outlier activities. However, other lines with "normal" activities for their allozyme also have transposable elements, often of similar size and in the same location, suggesting the altered activity does not result from the insertion. In addition, not all of the within allozyme activity variation is due to sequences tightly linked to Adh. For example, activity differences among two Fast lines which are outliers to the main Fast-high activity cluster (and have transposable elements 1.5 kb 3' to Adh), have been shown to be due to trans-acting modifier loci not closely linked to Adh, yet on the second chromosome.

In contrast, the low activity of one line (RI42) with a 5 kb copia element inserted 400 bp 5' to the start of the adult transcript is tightly linked and cis-dominant. This line also shows an altered developmental pattern of expression, with larval activity disproportionately reduced. This latter observation is notable since the larval transcript initiation site is nearly a kilobase farther from the site of insertion than from the beginning of the adult transcript. In addition to the genetic evidence, however, support for the copia insert as the cause of the altered activity comes from our own mRNA level studies that indicate the activity differences are correlated with mRNA level differences and transformation studies of Posakony and Maniatis that demonstrate that deletions of sequence from this 5' region affects larval activity to a greater extent than adult activity.

Thus, it is clear that alterations in flanking regions can have detectable effects. However, some variation in flanking sequences is not clearly associated with altered levels of adult ADH activity, yet may have significant effects. Only transposable elements showed a clear and significant departure from neutrality predictions. In particular, Waterson's homozygosity test indicated that each individual element (versus non-transposable element haplotypes)

was too rare. (This frequency observation has held up in our examination of D. melanogaster from populations throughout the world.) This departure could be accounted for by a high deletion rate (they insert and delete rapidly and never have a chance to accumulate in frequency at any one site). Alternatively, they may have a slight deleterious effect. Additional theoretical studies indicate the transposable elements (and probably the deletions) are deleterious although with selection coefficients only several times their rate of insertion. In addition, unlike base substitutions, sequence length variation is not observed to accumulate through evolutionary time.

While we saw only one clear instance of an apparent effect of a flanking transposable element on Adh expression, their apparent selective disadvantage may be due to subtle effects on the timing or tissue distribution of Adh expression for which only a few of the lines have been examined. The location of most of these elements 3' to Adh makes this perhaps not too likely. The deleterious nature of insertion/deletion variation and particularly transposable element insertion may have to do with effects on adjacent unknown genes or on chromatin stability in this region. Recall that eight of eleven transposable elements were located within a 230 bp region 3' to Adh, and two others just 3' to this site. We have been able to demonstrate that this apparent "hot spot" is also a DNAse I hypersensitive site in intact chromatin, sites found primarily at the 5' end of genes and implicated as being important for proper gene expression. The copia insert 5' to Adh may also be located in a DNAse I hypersensitive site. The significance of these observations remains obscure but one could speculate that preferential sites for transposable element insertion are also sites important in some aspect of gene control and/or chromatin structure, the implications of which would be substantial.

PLANS FOR SUBSEQUENT YEAR: The project will be concluded with the analysis of Adh messenger RNA levels in the 49 lines. This will allow the determination of the proportion of the quantitative variation of Adh activity that can be attributed to variation in transcriptional activity and/or messenger RNA stability. Some effort will be devoted to the further analysis of the molecular genetic basis of quantitative variation in gene expression at other loci that can be readily assayed.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The assessment of risk to the human population from exposure to environmental mutagens depends on a solid understanding of population genetics. The potential significance of insertion/deletion variants (particularly transposable elements) in and outside transcriptional units is unclear but the question is approachable. These results contribute to the understanding of domains responsible for proper gene expression and define genetic lesions that can upset that regulation.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61019-04 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Collaborative Protein Sequencing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Steven S.-L. Li	Research Geneticist	LG, NIEHS
Others:	Farida S. Sharief	Biologist	LG, NIEHS

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A glycoprotein exhibiting immunological and enzymatic activities of human prostatic acid phosphatase has been purified and amino-terminal sequences of both prostatic acid phosphatase and glycoprotein were found to be different.

A human prostate antigen was purified from both prostate gland and seminal plasma and its physico-chemical properties characterized.

Four ribonucleases have been purified to homogeneity and characterized from human seminal plasma. The seminal RNase III was found to be a prostate-specific enzyme, whereas others are similar to serum RNases.

Amino acid sequence of human dihydrofolate reductase has been determined, and the primary structure information is also helpful to understand the structure-function relationship of dihydrofolate reductase.

The primary structure information of protein is very important in elucidating the fundamental biological function. The collaborative research of protein sequencing will provide expertise so that fast and accurate information can be obtained for cloning and identification of eukaryotic genes.

PROJECT DESCRIPTION

PROBLEM: Collaborative protein sequencing: prostatic acid phosphatase, prostate antigen, ribonucleases and dihydrofolate reductase from human.

OBJECTIVES: The information on the primary structure of proteins is very important in elucidating the structure-function relationship of proteins. The collaborative research of protein sequencing will provide expertise so that fast and accurate information can be obtained for cloning and identification of eukaryotic genes.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: a) Prostatic acid phosphatase and prostate antigen: The prostate cancer is second-largest killer among men, after lung cancer. Since elevated activity of serum acid phosphatase is a combination of many isozymes from various tissues and blood cellular components, several immunoassays based on the immunologic specificity of prostatic acid phosphatase (PAP) have been developed as a diagnostic marker for the detection of PAP levels in serum from patients with prostate cancer. Another prostate-specific antigen (PA) has been purified and is being developed as a new marker for prostate cancer. In order to determine the complete primary and antigenic structures as well as to study the regulation of their expression, the partial amino acid sequences of both human PAP and PA will be determined for the synthesis of oligonucleotides used as probes to clone and identify their genes.

b) Ribonucleases: The levels of RNase activity in human serum and urine appears to be indicative of a disease state, including the prostate cancer. Since the secretion of the prostate gland contributes a major portion of seminal plasma, different human RNases isozymes were purified and characterized biochemically and immunologically in order to determine the number of different RNase isozymes (genes).

c) Dihydrofolate reductase: Since human DHFR is an important target enzyme for developing chemotherapeutic agents, knowledge of the detailed structure and the binding properties of this enzyme will be very helpful in designing more effective antifolate drugs and in understanding the selective inhibitor binding effects between host and parasite reductases. The amino acid sequence of human DHFR will also provide a direct evidence for conclusive identification of human DHFR functional genes.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: a) Prostatic acid phosphatase and prostate antigen: In collaboration with Dr. T.-M. Chu of Roswell Park Memorial Institute, two prostatic acid phosphatase isozymes (PAP-I & PAP-II) and a glycoprotein (GP) which is immunochemically and biologically related to PAP, have been purified and partially characterized from human seminal plasma. PAP-I possess two subunits of 50,000 each in comparison with subunits of 55,000 for PAP-II. The GP has an apparent molecular weight of 45,000. The purified GP was shown to exhibit a weak, but significant, acid phosphase activity. Amino acid

compositions, peptide maps and carbohydrate contents of PAP-I, PAP-II and GP have been obtained. Amino-terminal sequences of PAP-I and GP have also been determined. These chemical data as well as immunological results demonstrate that PAP-I and PAP-II are different human PAP isozymes and that the GP represents a distinct glycoprotein which shares some common enzymatic and antigenic characteristics with PAP. The prostate antigen has been purified from prostate gland and seminal plasma, and the antigen from both sources appears to be identical with respect to their molecular weight, isoelectric points, amino acid compositions and carbohydrate contents. In Dr. T. M. Chu's laboratory monoclonal antibodies prepared against PAP and PA have been obtained and immunotoxins using ricin and abrin are being developed to cure the human prostate cancer.

b) Ribonucleases: Four RNase isozymes have been purified and partially characterized from human seminal plasma. The molecular weights of RNase I, II, III and IV are 78KD, 16KD, 13KD, and 5KD, respectively, and they were also found to contain different amino acid compositions. RNase I, II, IV have a higher affinity for poly (C) over poly (U), whereas RNase III preferentially hydrolyzes poly (U) over poly (C). The antiserum against prostate-specific RNase III showed no immunological cross-reation with other RNases of human origin. Immunohistochemical study also demonstrates that RNase III is expressed only in human prostate.

c) Dihydrofolate reductase: Using 4.5mg of protein purified by Drs. B. A. Domin and Y.-C. Cheng, about 75% of the 186 amino acids have been positively identified by Edman degradations, and the sequence of remaining residues have been deduced from the amino acid compositions of isolated peptides and the sequence homology with those of other vertebrate DHFR enzymes. A comparison of human DHFR sequence with that of mouse L1210 enzyme indicates 23 amino acid differences, and these structural differences have correlated with their kinetic and functional properties.

FUTURE RESEARCH PLANS: 1. Acid phosphatase isozymes from other human tissues such as pancreas, spleen and liver will be purified and compared exzymatically and immunologically with those of prostatic acid phosphatase isozymes. Amino-terminal sequences for all acid phosphatase isozymes purified will be determined, and some of their peptides will be isolated and sequenced.

2. Amino-terminal sequence and partial sequence of some peptides purified from human prostate antigen will be determined.

3. Amino-terminal sequences and amino acid compositions of isolated peptides from different human RNases will be determined and compared with the known sequences of other RNases.

4. The 3-D structure of human DHFR enzyme will be illustrated by computer molecular graphics and the interaction of the enzyme active site with various drugs/inhibitors may also be studied by molecular dynamics.

PUBLICATIONS

Lin, M.F., Lee, C.L., Li, S.S.-L., and Chu, T.M.: Purification and characterization of a new human prostatic acid phosphatase isozyme. Biochemistry 22: 1055-1062, 1983.

Lin, M.F., Lee, C.L., Sharief, F.S., Li, S.S.-L., and Chu, T.M.: Glycoprotein exhibiting immunological and enzymatic activities of human prostatic acid phosphatase. Cancer Research 43: 3841-3846, 1983.

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Pan, Y.-C.E., Domin, B.A., Li- S.S.-L., and Cheng, Y.-C.: Studies of amino-acid sequence in hydrofoalte reductase from a human methotrexate-resistant cell line KB/6b: structural and kinetic comparison with mouse L1210 enzyme. Eur. J. Biochemistry 132: 351-359, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61021-03 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Molecular Analysis of the cut Locus of D. melanogaster

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*

PI:	Joseph W. Jack	Senior Staff Fellow	LG, NIEHS
Other:	Willie Gibson	Research Chemist	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are interested in knowing how cells of a single organism can differentiate to form specific tissue types. We have chosen to address one aspect of the question by learning how one gene, the cut locus of Drosophila melanogaster, is expressed differently in different tissues of the fly.

We have now cloned DNA sequences representing the entire gene, which encompasses 150 kb or more of DNA. A large number of mutants have been analyzed. We find that the deletion of sequences in the leftmost part of the gene cause phenotypic effects primarily in the legs, while deletion of or insertions into sequences slightly to the right cause effects primarily in the wings. Mutations in a third region 70 - 80Kb away at the rightmost end of the gene prevent expression in both legs and wings and cause lethality of the flies when homozygous.

The availability of tissue specific mutants of a gene afford the opportunity to experiment to find out how the gene normally operates in tissue specific ways. We are currently studying the transcriptional activity of the cut locus and the DNA structure of normal and mutant alleles with the intention of finding out what causes the mutations and how the activity is altered.

We now know that many of the cut mutants are insertions of retrovirus-like sequences into the cut locus DNA, and we are interested in understanding the affect of these sequences on gene activity. Some of these mutations are suppressible and will be useful in determining how a mutation caused by a retrovirus-like sequence can be suppressed.

PROJECT DESCRIPTION

MAJOR FINDINGS AND PROPOSED COURSE: In the past year we have completed the cloning of all or most of the DNA comprising the cut locus. The gene is very large, containing 150 kb or more of DNA. Despite its extraordinary size, cut sequences tested have been restricted to a single band on a polytene chromosome. This observation relates to the question of how genes are organized on chromosomes. It has long been noted that complementation groups in Drosophila are frequently associated in a one-to-one fashion with polytene chromosome bands. However, the question of whether these bands represent functional units remains an open question. In this context it is remarkable that although the average band contains about 20-30 kb of DNA, the entire 170 kb of cut locus is limited to a single band.

A number of tissue specific mutations have now been analyzed. The analysis of several deletions demonstrates that one segment of the gene is necessary for cut expression in the legs and another for expression in the wings. Previous genetic experiments have localized a group of lethal cut mutants to DNA separate from the tissue specific mutants, thus defining three regions of the gene. Thirteen mutations that cause loss of tissue on the wing margin have been studied. These thirteen have no effect on the legs and are all associated with insertions into the wing specific sequences of cut. Seven of these mutations are transposable elements similar in structure to retroviruses. The others are of a size consistent with their being transposable elements as well.

In collaboration with Dr. Johng Lim we have studied the nature of cut locus mutations generated in a mutable strain of flies. These mutations are of the wing specific and lethal varieties. We find that most of these mutations are caused by the insertion of the retrovirus-like transposable element gypsy into cut locus DNA. Thus, the gypsy element apparently transposes exceedingly frequently in the mutable strain, causing mutations at least in the cut locus. Many mutations associated with the insertion of gypsy elements at cut and other loci are known to be suppressed by the mutation of another gene, suppressor of Hairy wing. We are interested in knowing what effect transposable elements exert on genes to prevent their normal functioning. The gypsy element insertions are particularly useful because of the opportunity to suppress the effect of the mutation. By characterizing the location of gypsy elements with respect to cut locus transcripts, their effect on transcription, and their ability to be suppressed, we expect to learn more about the effect of the elements on gene activity and the suppression of that effect. So far we know that five of six wing specific mutants generated from the unstable strain and tested are suppressible, and the sixth is enhanced by the suppressor. Five of five lethal mutations tested are unaffected.

In addition to the mutations caused by the insertion of gypsy into cut locus, many of the mutants from the unstable strain are conversions of a wild type allele to the ct^6 allele. We have entered a collaboration with Dr. Lim and Dr. Burke Judd to find out how the ct^6 allele is transmitted from one chromosome to another.

With respect to the transcriptional activity of cut, a 1.8 kb transcript has been detected with probes from the wing specific region of cut. In the coming year we expect to study the transcription of the gene in some detail using probes from the entire gene.

We have now located and begun to analyze a number of lethal mutations to complete our knowledge of the organization of various types of mutations within the gene.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Our analysis of the cut locus is aimed at understanding how genes are regulated during the development of a higher organism and how the process of regulation can be altered by various types of mutations. The analysis of the changes in cut locus activity caused by retrovirus-like sequences will be important for understanding how such sequences affect genetic activity. This understanding will be important since retroviruses are known to alter the behavior of cellular genes, in some cases giving the altered cells the potential to form tumors.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61022-03 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Population Genetics of Transposable Elements

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. H. Langley	Research Geneticist	LG, NIEHS
Others:	Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

Dr. Norman Kaplan and Dr. Thomas Darden
Biometry and Risk Assessment Program

Dr. Brian Charlesworth
University of Sussex
England

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 - ☐ (a1) Minors
 - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The evolutionary history of Drosophila transposable elements was studied. More than ten different families of transposable elements identified in D. melanogaster were found also in related Drosophila species. The single exception was the P element that was found only in stocks of D. melanogaster recently collected from wild populations. It appears that most transposable elements are stable components of the host genome. The single exception among those studies is the P element that may have recently invaded.

Theoretical studies of the extinction process of a transposable element family in a Mendelian host population were carried out. The impact of mutation within the transposable element to a defective copy was examined. Although values of various parameters can have large effects on the extinction time, it is generally true that mutation to defective elements accelerates the extinction process. This provides a scenario for the evolution of the P element in Drosophila melanogaster that rearranges to putative defective copies at a high rate.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: The existence of transposable elements (TE's) has been documented in a number of both prokaryotic and eukaryotic organisms. There is a growning body of evidence linking transposable elements with spontaneous mutations in Drosophila and other organisms. Little is known, however, of the occurrence and effects of these elements in natural populations. How these elements have evolved is also unclear.

OBJECTIVES: We are seeking to accumulate data on the natural history of this class of DNA parasites in Drosophila melanogaster and in related species. While some transposable element insertions cause visible mutations of major effect, the effects on the host of the vast majority of TE's is not known. Another important question concerns the means by which TE's have come to be in the Drosophila genome. Have they arisen within the Drosophila lineage through evolutionary changes generating new TE families, either from unique sequence DNA or from other TE families? Or have Drosophila TE's been transmitted horizontally into Drosophila from other evolutionary lineages? What happens to an element when mutational changes occur within the element itself?

EXPERIMENTAL DESIGN AND SCIENTIFIC JUSTIFICATION: The family of transposable elements in Drosophila known as copia-like elements bear a strong resemblance to vertebrate retrovirus sequences, the insertion of which has been implicated in oncogenesis. This and other families of transposable elements in Drosophila, such as the P element, are responsible for spontaneous mutations. The identification, cloning and characterization of these elements from D. melanogaster affords us the opportunity, using molecular and cytological techniques, to learn about the population biology of the elements and their effects on the organisms they inhabit. Both experimental and theoretical tools have been used to approach these problems.

One approach to the question of origin is to look for homologies between cloned middle repetitive sequences from Drosophila melanogaster and the DNAs of related species. If TE's are capable of horizontal transmission, the distribution of homologies may not follow the phylogeny of the host.

The question of the effect on the host of TE's in the absence of obvious mutations can also be tested. Currently, the hypothesis that these elements may have a mildly deleterious effect in nature is being tested. Drosophila melanogaster chromosomes, genetically isolated from a natural population, are probed with cloned TE's in situ. Comparisons are made for each element between the number present on the X chromosome versus the number on the autosomes. One would expect greater selection in nature against deleterious effects in the X chromosome.

In addition to experimental approaches, theoretical studies of the extinction process of a particular class of transposable element that rearranges to putative defective copies at a high rate were also carried out.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: Nineteen cloned middle repetitive sequences from Drosophila melanogaster were used as probes to search for homologies to DNAs of two closely related species and to three more distantly related species of D. melanogaster. With the single exception of the P element, multiple copies of all these TE's from Drosophila melanogaster were found in the two closest relatives of D. melanogaster, and many of them showed homology to more distantly related species as well. This phylogenetic distribution suggests a long and stable evolutionary history. The only exception to this distribution was the P element which occurred solely in the D. melanogaster stocks recently collected from the wild. A likely hypothesis is that the P-element has invaded D. melanogaster at a comparatively recent date.

A mathematical model of the extinction process of an element such as the P-element was done. The model incorporated mutation within the element. (The P-element has considerable genetic variation in the copies studied so far). This model suggests that if an element does not become extinct in the first few generations, then the average copy number of the wild type increases quickly in the early stages of the element's evolution, and then decreases slowly as the average copy number of the mutant starts to grow. After a period of stabilization, the wild type disappears from the population leaving only mutant copies, transposition stops and the mutant eventually deletes out of the population. The model supports the hypothesis that elements characterized by mostly defective copies - as P elements - may have recently invaded, and can be expected to have a shorter, less stable evolutionary history. The model, as well as experimental results, also suggests that elements with mostly functional copies, e.g. copia-like elements, are ancient and have long, stable evolutionary histories.

PLANS FOR SUBSEQUENT YEARS: Work is now in progress to study the impact on a natural population of D. melanogaster of several different types of transposable element families by comparing the copy number on the X chromosome to the number on the autosomes. If the hypothesis is correct that they are mildly deleterious, there should be fewer copies on the X than on the autosomes. We are also investigating the accumulation of transposable elements within chromosomal inversions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Mounting evidence suggests that many "spontaneous" mutations are due to insertion of transposable elements. The insertion of transposable-element-like retrovirus sequences are implicated in oncogenesis. The understanding of the population biology of these chromosomal parasites will be essential in evaluating population risk to both mutagenesis and cancer.

PUBLICATIONS

Langley, C.H., Brookfield, J.F.Y., and Kaplan, N.: Transposable elements in Mendelian populations. I. A theory. Genetics 104: 457-471, 1983.

Montgomery, E.A., and Langley, C.H.: Transposable elements in Mendelian populations. II. Distribution of three copia-like elements in a natural population of Drosophila melanogaster. Genetics 104: 473-483, 1983.

Kaplan, N., and Brookfield, J.F.Y.: Transposable elements in Mendelian populations. III. Statistical results. Genetics 104: 485-495, 1983.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61023-02 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of Drosophila Germ Cell Determination

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Robert E. Boswell Staff. Fellow LG, NIEHS

Others: Steven A. Haneline Biological Laboratory Technician LG, NIEHS

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LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Usa standard unreduced type. Do not exceed the space provided.)*

It is a fundamental concept in developmental biology that the fate of embryonic cells is regulated by morphogenetic determinants localized in the ooplasm. In Drosophila, heterotopic transplantation experiments have conclusively demonstrated that cytoplasmic factors localized to the posterior pole plasm of the oocyte and embryo are requisite for the formation of pole cells, the primordial germ cells. However, the molecular nature of these cytoplasmic factors, the mechanism of localization within the ooplasm, and their mode of action in development are unknown. The genetic and developmental analysis of maternal effect mutants that affect pole cell formation in Drosophila melanogaster are intended to allow one to elucidate the mechanism of determination and how the determined state is maintained throughout development.

A detailed genetic and developmental analysis of one such grandchildless mutant, tudor, (tud) has been undertaken. The properties of mutations of the recessive maternal effect gene tud indicate that the gene product of the tudor locus is required for the proper determination of germ cells in Drosophila melanogaster. Specifically, the germ plasm of six different alleles of tud has been analyzed at the ultrastructural level, and it is found that different alleles contain different amounts of assembled polar granule material (a cytoplasmic organelle classically thought to be the germ cell determinants). The ability or inability to form germ cells correlates directly to the amount of assembled polar granule material observed in the germ plasm. For example, one allele produces polar granules approximately 1/3 the size of wild type polar granules and this allele produces fertile progeny. On the otherhand, alleles that produces no apparent assembled polar granule material in the germ plasm produce no fertile progeny. Therefore, mutations at the tudor locus disrupt the normal assembly of the germ plasm resulting in the failure to localize the germ plasm determinants to the posterior pole.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: It is a fundamental concept in developmental biology that the fate of embryonic cells is regulated by morphogenetic determinants localized in the ooplasm. In Drosophila, heterotopic transplantation experiments have conclusively demonstrated that cytoplasmic factors localized to the posterior pole plasm of the oocyte and embryo are requisite for the formation of pole cells, the primordial germ cells. However, the molecular nature of these cytoplasmic factors, the mechanism of localization within the ooplasm, and their mode of action in development are unknown.

OBJECTIVE: An understanding of the molecular mechanisms involved with germ cell determination would be a significant contribution to our understanding of developmental processes. The aim of this research then is to determine (1) the molecular nature of the determinants required for specifying the fate of a particular cell type (the primordial germ cells), (2) how these molecules specify and maintain the particular developmental commitment undertaken by these cells and (3) the means by which these informational molecules become localized to a discrete cytoplasmic site within the egg.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: The analysis of germ cell determination in Drosophila will require a combined genetic, developmental and molecular characterization of the process. The strategy is to isolate and characterize selected grandchildless loci. These loci will be characterized to determine the nature of the developmental lesion induced by disrupting the normal gene function. The available technologies (e.g. recombinant DNA, sequencing and antibodies) will be used with the intent of isolating the gene products and establishing their roles in germ cell determination.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: A new maternal effect grandchildless (gs) mutant, tudor, has been analyzed. New alleles of tudor were recovered by X-ray mutagenesis. The posterior pole plasm of oocytes and preblastoderm embryos was analyzed by transmission electron microscopy (TEM).

Homozygous tudor females produce embryos that lack pole cells and the adults of both sexes are sterile. The germ plasm produced by six different alleles of tudor has been analyzed ultrastructurally by TEM. Oocytes or embryos from each genotype contain different amounts of assembled polar granule material, such that they form a continuum from those that contain no apparent assembled polar granule material to types that contain polar granules approximately 1/3 the size of polar granules observed in the wild type oocyte or embryo. The ability or inability of any given allele to produce pole cells (the primordial germ cells) correlated directly with the amount of assembled polar granule material in the germ plasm. For example, the allele (tud^4) produces oocytes and embryos with polar granules approximately 1/3 the size of wild type polar granules and is fertile as a homozygote. However, all alleles containing polar granules less than 1/3 the size of wild type polar granules or no apparent polar granule

material are sterile as homozygotes. This indicates then, that mutations at the tudor locus disrupt the normal assembly of the germ plasm in a quantitative fashion, different alleles contain different amounts of polar granule material. It is postulated that the failure to properly assemble the germ plasm disrupts the localization of the germ cell determinants thereby resulting in a failure to form germ cells.

Like most maternal effect mutants studied to date, tudor is pleiotropic, and approximately 40% of the embryos from the homozygous gs females die during embryogenesis and exhibit segmentation pattern abnormalities. The remaining 60% of the embryos are phenotypically normal, but they are sterile due to a failure in germ cell formation. The severity of the segmentation pattern abnormalities and the sterility due to a failure in pole cell formation correlate with the amount of assembled polar granule material in the germ plasm. For example, $tudor^4$ is not grandchildless as a homozygote, but is grandchildless when heterozygous with tudor alleles containing reduced amounts of polar granule material in the germ plasm. This leaky allele expresses few, if any, of the segmentation pattern abnormalities observed in embryos derived from homozygous females of tudor alleles containing no or reduced amounts of polar granule material assembled in the germ plasm.

These properties of mutations at the tudor locus indicate that the gene product of the tudor locus is required for the determination of germ cells and for the normal pattern of segmentation in Drosophila melanogaster. In particular, the tud^+ gene product appears to be a component necessary for the proper assembly of the germ plasm.

Various X-ray induced alleles of tudor are chromosomal rearrangements. Deletion mapping taken together with the cytological mapping of the chromosomal rearrangements place the tudor locus in the right arm of the second chromosome within the polytene chromosome interval 57C8-9. Recently, a unique sequence segment of Drosophila melanogaster DNA cloned into the λ cloning vector Charon 4 has been found to be homologous to the polytene chromosome interval 57C. Using standard molecular and cytogenetic techniques we have determined the position of this cloned segment of Drosophila melanogaster, DNA relative to the tudor locus. We have walked approximately 60 kbp from the site of entry towards the tudor locus.

PLANS FOR SUBSEQUENT YEARS: New alleles of tudor and loci flanking the tudor locus will be obtained using a variety of mutagens (X-ray, diepoxybutane, N-ethyl-N-nitroso-urea, ethylmethane sulfonate). The pole plasm of oocytes and preblastoderm embryos will be analyzed by transmission electron microscopy (TEM). Standard molecular techniques will be used to obtain recombinant DNA clones containing the tudor structural gene.

The genetic analysis of tudor and other grandchildless loci will allow one to study the cytological consequences of genetic lesions leading to grandchildless phenotype. These studies will require TEM of the pole plasm of stage 13-14 oocytes as well as preblastoderm embryos. Pole cells of blastoderm embryos will

also be analyzed cytologically. Scanning electron microscopy (SEM) will be used to analyze any morphological defects in developing embryos that may be associated with the abnormal segmentation. Segmentation defects can also be studied using acetylcholinesterase whole mounts and plastic sections of whole embryos. The segment defects will also be studied using Hoyer's mounts to identify the cuticle pattern defects.

The chromosomal walk undertaken to isolate the tudor structural gene will be continued. Chromosome rearrangements associated with the tudor locus will serve as molecular landmarks to define a region of the genome that must be intact for the proper expression of the tudor locus. Furthermore, we have several small deficiencies (approximately 5 chromosomal bands) that define the left and right most boundaries of the tudor locus.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A major interest within the institute is to understand the mechanisms involved in cellular determination and differentiation. Elucidation of the molecular events in determination is considered crucial to understanding medical problems such as cancer and aging.

PRIORITY NEEDS: The developmental analysis of tudor mutations will require ready access to a transmission and scanning electron microscope. A completion of the chromosomal walk to clone the tudor locus would most readily be accomplished with one more technical assistant.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61024-02 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Molecular Analysis of Suppressor-of-Sable Function in Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. A. Voelker	Research Geneticist	LG, NIEHS
Others:	D.-Y. Chang	Visiting Fellow	LG, NIEHS
	G. B. Wisely	Bio. Lab. Tech.	LG, NIEHS
	J. F. Sterling	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	2.8	1.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent findings in Drosophila have shown that 1) a significant proportion of spontaneous mutations are caused by insertions of mobile genetic elements, and 2) certain genetic suppressor systems are mediated through insertions of specific mobile elements. We are investigating the molecular mechanism of one such suppressor system: recessive mutations at the suppressor-of-sable [su(s)] locus suppress recessive mutations at the vermilion (v) locus that are caused by insertions of the mobile elements 412 and B104 (Project Number Z01 ES 61029-02 LG).

DNA sequences of su(s) have been cloned and are being characterized to determine the transcription orientation and the locations of the control and coding sequences. A primary goal of the work is to determine the protein product of su(s), a reduction or an absence of which effects suppression of several v alleles. Coding sequences from su(s) will be ligated into an expression vector to produce a fusion protein, against which antibodies can be produced. Antibodies against the su(s) portion of the fusion protein will be recovered and used as probes to identify the location and function of the su(s) protein product within the organism. The interaction of the su(s) protein with the v locus will be studied to determine how suppression is effected. By gaining an understanding of this phenomenon, we will learn if this type of suppression mechanism is an adaptive feature of Drosophila to deal with mutations caused by mobile element insertions.

PROJECT DESCRIPTION

PROBLEM: Recent findings have shown that a significant proportion of spontaneous mutations in Drosophila are caused by insertions of mobile genetic elements, and that certain genetic suppressor systems are mediated through insertions of specific mobile elements. We are investigating the molecular mechanism of one such suppressor system: recessive mutations at the suppressor-of-sable [su(s)] locus suppress recessive mutations at the vermilion (v) locus that are caused by insertions of the mobile elements 412 and B104.

OBJECTIVES: A primary goal of the work is to determine the protein product of su(s), a reduction or an absence of which effects suppression of several v alleles. Coding sequences from su(s) will be ligated into an expression vector to produce a fusion protein, against which antibodies can be produced. Antibodies against the su(s) portion of the fusion protein will be recovered and used as probes to identify the location and function of the su(s) protein product within the organism. The interaction of the su(s) protein with the v locus will be studied to determine how suppression is effected. By gaining an understanding of this phenomenon, we will learn if this type of suppression mechanism is an adaptive feature of Drosophila to deal with mutations caused by mobile element insertions.

METHODS EMPLOYED:

1) Duplication/deficiency mapping and genetic complementation tests will be used to analyze the mutants recovered from a lethal saturation mapping of the su(s) region.

2) The P-element transposon-tagging technique has been used to clone both su(s).

3) Conventional endonculease restriction mapping is being used to characterize the structure of wild type and mutant DNA sequences.

4) Northern blot analyses of polyA$^+$ RNA's are being used to map the extents of coding sequences.

5) M13 single-stranded probes are being used to identify the direction of transcription.

6) S1 nuclease-type mapping methods are being used to locate the intron and exon components of the coding sequences.

7) The structure and function of the su(s) protein product are completely unknown; therefore, a fusion protein expression vector system will be used to express and recover a hybrid protein from which to make and recover antibodies which will be used to recover and localize the entire protein.

MAJOR FINDINGS AND PROPOSED COURSE:

1) The P-element transposon tagging technique has been used to clone DNA sequences of su(s).

2) Using wild type DNA sequences of su(s) as probes, the restriction maps of 23 su(s) mutant alleles have been analyzed.

 a) All of seven spontaneous mutations contain insertions of non-su(s) DNA sequences; many of these inserted DNA's are mobile genetic elements.

 b) None of nine X-ray or EMS-induced mutations contain altered restriction patterns;

 c) All of five hybrid dysgenesis-induced mutations contain insertions of non-su(s) DNA sequences.

 d) Two of two DNA transformation-induced mutations contain insertions of non-su(s) DNA sequences.

3) A 3.5-4 kb polyA$^+$ message is homologous with sequences spanning approximately 8 kb of DNA from the su(s) region; this suggests the presence of sizeable introns.

4) Appropriate subclones have been constructed so that S1-type mapping experiments can be carried out to localize the intron and exon sequences of the su(s) gene.

5) The direction of transcription of su(s) is distal to proximal (telomeric → centromeric);

6) An exon sequence of 0.5-1 kb from the su(s) region will be ligated into the expression vector pMR100 to produce a su(s)-B galactosidase fusion protein. The fusion protein can be precipitated with commercially available anti-B-galactosidase antibodies. The precipitate can be injected into rabbits to produce antibodies which can be fractionated to recover those specific for the su(s) component of the fusion protein. These su(s)-specific antibodies can be used as probes for the structural location and function of the su(s) protein product.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is becoming apparent that mobile genetic elements are responsible for many so-called spontaneous mutations. Since most mutations are harmful, such mutations contribute to the genetic loads of populations and in humans these mutations become causes of genetic diseases. Thus, an understanding of how these mobile genetic elements cause mutations and how an organism may evolve systems to suppress these mutations offers the prospect of minimizing the effects of such mutations or perhaps eventually elimination them.

PUBLICATIONS

Voelker, R.A., Chang, D.-Y., Huang, S.-M., and Wisely, G.B.: Cloning and molecular characterization of the suppressor-of-sable [su(s)] gene from Drosophila. Abstract. Genetics 107: 111-112, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61025-02 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

DNA Sequence Variation in the Dopa Decarboxylase Region of Drosophila

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles F. Aquadro	Staff Fellow	LG, NIEHS
Others:	Susan F. Deese	Bio. Lab. Tech.	LG, NIEHS
	Charles H. Langley	Research Geneticist	LG, NIEHS

COOPERATING UNITS *(if any)*

Dr. C. Laurie-Ahlberg, Associate Professor of Genetics
North Carolina State University, Raleigh, North Carolina

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project has been to examine the patterns and levels of naturally occurring DNA sequence variation in and around a dense cluster of lethal-mutable genes in Drosophila melanogaster. Of particular interest was the affect of sequence alternations within and flanking the gene encoding the enzyme dopa decarboxylase (Ddc). No clear patterns between sequence variants and Ddc expression were observed, despite two-fold variation in DDC enzyme activity among the 46 lines examined. Located adjacent to the 30 - 40 kilobases of DNA sequence containing the dense cluster of eight lethal-mutable genes (including Ddc) is an approximately 40 kilobase region in which, at most, one gene appears to be located. We were interested in levels of sequence variation, particularly deletions and insertions (including transposable elements), in this latter region. If mutations occur randomly throughout the genome, yet persist for any length of time only in regions where they produced little or no deleterious effect, we should see less variation in the dense cluster of genes than in the adjacent 40 kb region. Examination of DNA sequence variation by restriction mapping in this 80 kilobase region has revealed the opposite pattern. Virtually no variation was observed in the genetically sparse region while numerous insertions/deletions in the region containing the dense cluster of genes. While we cannot rule out the selective maintenance of variation in the gene cluster, these results raise the possibility of increased insertion/deletion mutational activity in transcriptionally active regions although unknown constraints may exist in the nonvariable region. We are testing these hypotheses in several other regions of the Drosophila genome.

PROJECT DESCRIPTION

PROBLEM: Our understanding of the frequency, nature of, and significance of naturally occurring DNA sequence variation is in its infancy. Little is known of the extent of the generality of transposable element insertion site specificity such as that seen in our examination of the alcohol dehydrogenase region of Drosophila melanogaster. In addition, we have little knowledge of the extent to which natural selection maintains DNA sequence variation and nonrandom associations of sequence variants at linked genes. All of this information is important if we are to understand the significance of the sequence diversity apparently present in most organisms, including humans, and if we are to be able to assess the long-term significance of mutational damage.

OBJECTIVES: The objectives are to determine (1) What levels of DNA sequence variation occur normally. (2) Where, relative to structural genes or other regions of defined function, these sequence alterations occur. (3) Whether transcriptionally active regions are more variable than nontranscribed regions. (4) The extent and origin of nonrandom associations among DNA sequence variants along the chromosome.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: Information concerning the extent, location and significance of naturally occurring DNA sequence variation is central to the assessment of the significance of mutagen exposure. In addition, studies of apparently normal individuals provides important information concerning the nature and size of the "target" for mutagenesis and the types of variation that can and cannot be tolerated. A population genetic approach is particularly valuable for these types of problems since effects of an extremely subtle, yet significant, level of natural selection can often be detected since we effectively examine the variant's significance over thousands or hundreds of thousands of generations.

We have chosen to study Drosophila melanogaster from natural populations and to focus on the region around the structural gene encoding the enzyme dopa decarboxylase (Ddc) for several reasons. Foremost is the fact that this is an extremely well studied region in terms of genetics, biochemistry and molecular genetics. The dopa decarboxylase structural gene is located on chromosome II. Ddc catalyzes the decarboxylation of dopa to dopamine and 5-hydroxytryptophan to seratonin. Enzymatic activity is required for cuticle hardening, female fertility, tissue specificity and proper functioning of the central nervous system. Ddc is located in the middle of a large dense cluster of 17 genes and thus provides us an ideal opportunity to examine sequence variation in a functionally related gene cluster. In addition, two quite different regions appear to be juxtaposed: one region containing Ddc and at least 6 to 7 additional genes (identified as lethals and regions encoding messenger RNAs), some of which appear to be functionally related and packed within roughly 30 kb of DNA sequence; and a second region immediately adjacent that contains at most one identified gene in well over 40 kb of sequence. Importantly, over 100 kb of this entire Ddc region had been cloned by J. Hirsh who kindly has provided us with the clones which we can use to probe the entire region, including the

transcribed and nontranscribed portions. Studies of this magnitude are generally not possible in humans due to the large number of small repeat sequences scattered throughout the mammalian genome making it difficult to obtain probes to completely assay large regions of the mammalian genome in the same way.

Forty-six genetically isolated second chromosomes of D. melanogaster were reared and nuclear DNA isolated. Restriction maps were constructed for an 80 kb region around Ddc and compared for evidence of nucleotide substitutions and insertion/deletion variation. Gene expression at the Ddc gene was assayed by examining enzymatic activity. We were not able to assay gene expression at the other genes surrounding Ddc since they are at present known only by their lethal phenotype and presence of messenger RNA (the precise limits of which have not yet been defined).

RECENT ACCOMPLISHMENTS: Forty-six second-chromosome lines of Drosophila melanogaster isolated from five natural populations were surveyed for restriction map variation in an 80 kb region surrounding the gene encoding dopa decarboxylase (Ddc). Eighty-four restriction sites were scored, 24 of which were polymorphic. While the polymorphic sites occur throughout the 80 kb region, those that are highly variable are clustered within a 40 kb region containing Ddc and a dense cluster of functionally related lethal genes. Five large (1.5-5 kb) inserts, one small insert (250 bp) and four small (100-200 bp) deletions were observed to be similarly localized to the 40 kb region. Surprisingly, no insertion/deletion variation was observed in the 40 kb region that appears to contain at most one gene. Significant nonrandom associations were observed among restriction sites in approximately the same 40 kb region but drop off 5' to the gene cluster. That all measures of variation show a marked decrease at virtually the same location (the break between the functionally related gene cluster and the genetically sparce region) suggests that the patterns are not due simply to chance and may reflect selection for favored combinations of alleles in this cluster of genes associated with female fertility and cuticle formation.

We have also assayed adult DDC activity in these lines. Surprisingly, two lines with 5 and 1.5 kb inserts within an intron of and at the 5' end of Ddc, respectively, show completely normal adult activities. Unlike our results for the alcohol dehydrogenase region of these same lines, no consistent pattern of association between level of Ddc activity and restriction site haplotype is apparent, although two-fold variation in activity among lines is observed within this sample of 46 lines.

PLANS FOR SUBSEQUENT YEAR: We were surprised by the apparent correlation between transcribed regions and DNA sequence variation. These results, taken together with those of our work on the alcohol dehydrogenase region of D. melanogaster (described under a separate project), suggest the need for examination of additional regions of the genome that are well characterized. Unfortunately, we have reached the limits of the well characterized sequences of the Ddc region. We do, however, plan some transcription mapping in the region that appears to be genetically depauperate.

Aquadro has also initiated an analysis of DNA sequence variation in two large, well characterized regions of the genome to address specifically the questions of an association between insertions and transcriptionally active regions, higher levels of nucleotide sequence variation in gene regions and a correlation between nonrandom associations and clusters of functionally related genes. The first region is the 315 kb region of chromosome III of D. melanogaster running from the rosy (xanthine dehydrogenase) gene to the gene encoding acetylcholinesterase (ace). This region is one of the best characterized large regions from the viewpoint of fine structure genetics, genomic DNA structure and location of transcripts. Contained within the 315 kb are about 14 bands in the polytene chromosome and sequences associated with at least twelve complementation groups (including the genes rosy and ace). The entire region has been cloned and the clones have been made available for our use by Dr. W. Bender. Aquadro and Deese have made a new large collection from a local natural population of D. melanogaster and genetically made the appropriate region of the third chromosome homozygous for independent chromosomes. Genomic DNAs have been prepared from 87 lines, cut by appropriate restriction endonucleases, run on gels and Southern blotted onto zetabind nylon filters. We can probe these filters repeatedly tens of times, each time washing off the previous probe, thus allowing us to "walk" down the genome examining patterns of DNA sequence variation. We have probed approximately one third of the 315 kb region but have only begun to score the variation. It is clear however, from a preliminary glance at the autoradiographs, that some regions are strikingly conserved while others show reasonably high levels of nucleotide polymorphism. Surprisingly, since we have examined a major transcriptionally active section of the region, we have seen a relatively low frequency of insertion/deletion variation. We have a substantial amount of mapping of restriction sites to do, however, before we can be confident of that result. We also plan to extend this analysis to a second, larger region on the third chromosome: the bithorax complex. This region is again the site of intensive study concerning its genetics and molecular biology and is of particular interest here due to its complex nature of interacting alleles, large transcripts (70 kb or more), and role in segmentation pattern formation in developing flies. Again, we have obtained the clones to probe a major portion of this complex for naturally occurring DNA sequence variation using the same lines as for the rosy to ace region. The results from these regions, direct comparison of the spatial organization of the variation with the location of known genes and transcripts, examination of possible effects of the variation on adjacent genes (assessed by enzyme activity and/or messenger RNA levels), and patterns of nonrandom association of variants should provide significant insights into the forces guiding the occurrence, location, maintenance and significance of DNA sequence variation in transcribed and nontranscribed regions of the genome.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The study of naturally occurring DNA sequence variation provides information on background levels of variation, information vital to the assessment of the significance of mutagen exposure. The results also contribute significantly to determining the "target" for mutagenesis and the types of variation that can and cannot be tolerated.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61026-02 LG

PERIOD COVERED
October 1, 1983 to December 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mammalian Mitochondrial DNA Variation and Evolution

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Charles F. Aquadro	Staff Fellow	LG, NIEHS
Others:	Norman Kaplan	Mathematician	BRAP, NIEHS
	Kenneth J. Risko	Mathematician	BRAP, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project was to determine the major features of mammalian mitochondrial DNA sequence variation and evolution as it reflects on the underlying mechanisms of mutation. Human mitochondrial DNA sequence comparisons were made, resulting in the detection of high frequencies of multiple (repeated) nucleotide substitutions and insertions/deletions. Two substitution biases were apparent, one favoring transitions by a factor of 32:1 over transversions and the other favoring a high rate of turnover of purines relative to pyrimidines on the heavy strand of mtDNA. Their occurrence in coding and noncoding regions as well as ribosomal RNA and transfer RNA genes suggests that these phenomena may result from biases in the mutational pathways since it is unlikely that similar selective constraints would exist in these functionally very different regions. We have also modeled the dynamics of the substitution process in mammalian mtDNA. We have studied the temporal behavior of several quantities and compared the model's predictions with estimates obtained from recent mtDNA sequence data for an increasingly divergent series of primates, the mouse and the cow. Our results are consistent with the hypothesis that the decrease in the proportion of transitions observed as divergence increases is a consequence of the highly biased substitution process. In addition, our results support the hypothesis that while a portion of the mtDNA molecule evolves at an extremely rapid rate, a significant portion of the molecule is under strong selective constraints related to conservation of protein sequence and transfer RNA secondary structure.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: Little is known of the nucleotide substitution process in mammalian mitochondrial DNA (mtDNA), the reasons for an apparently high rate of substitution, or the existence and/or nature of specific selective constraints acting on the mammalian mitochondrial DNA molecule.

OBJECTIVES: This project addresses the nature and extent of DNA sequence variation in human mitochondrial DNA as well as the properties of the nucleotide substitution process that govern the generation of sequence diversity in mammalian mitochondrial DNA. Do substitution biases reflect mutational biases or natural selection? Can we reconcile the conservation of size and genetic arrangement of mammalian mtDNA with the high rate of nucleotide substitution?

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: Our lack of understanding of the extent and nature of nucleotide substitution biases in human and mammalian mtDNA make the evaluation of the effect of mutagens on mtDNA difficult. Since mammalian mtDNA are autonomously replicating, distinct organelle genomes, it would be unwise to extrapolate nuclear mutation frequency spectra to mtDNA without first evaluating differences that may exist between the two systems. Our approach has been to analyze naturally occurring mtDNA sequence variation within humans and between humans and related primates, and the mouse and the cow. This provides us with the types and frequency of variation that occurs naturally. Our approach has been to statistically analyze published human and mammalian mtDNA sequences. Mathematical models of mtDNA sequence evolution have also been constructed and their predictions studied and compared with observed data.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: We analyzed nucleotide sequence variation in an approximately 900-base pair region of the human mitochondrial DNA molecule encompassing the heavy strand origin of replication and the D-loop. Our analysis focused on nucleotide sequences available from seven humans. Average nucleotide diversity among the sequences is 1.7%, several-fold higher than estimates from restriction endonuclease site variation in mtDNA from these individuals and previously reported for other humans. This disparity is consistent with the rapidly evolving nature of this noncoding region. However, several instances of convergent or parallel gain and loss of restriction sites due to multiple (repeated) substitutions were observed. In addition, other results suggest that restriction site (as well as pairwise sequence) comparisons may underestimate the total number of substitutions that have occurred since the divergence of two mtDNA sequences from a common ancestral sequence, even at low levels of divergence. This emphasizes the importance of recognizing the large standard errors associated with estimates of sequence variability, particularly when constructing phylogenies among closely related sequences.

Analysis of the observed number and direction of substitutions revealed several significant biases, most notably a strand dependence of substitution type and a 32-fold bias favoring transitions over transversions. The occurrence of these substitution biases in coding and noncoding regions as well as ribosomal

and transfer RNA genes suggests that these phenomena may result from biases in the mutational pathways since it is extremely unlikely that similar selective constraints would exist over such functionally different regions. The results also revealed a significantly nonrandom distribution of nucleotide substitutions and sequence length variation. Significantly more multiple substitutions were observed than expected for these closely related sequences under the assumption of uniform rates of substitution. The bias for transitions has resulted in predominantly convergent or parallel changes among the observed multiple substitutions. We found no convincing evidence that recombination has contributed to the mtDNA sequence diversity we have observed.

We have also studied the dynamics of the substitution process in mammalian DNA using a continuous-time, four-state Markov chain model. We have drawn on published sequences from humans, an increasingly divergent series of primates, the mouse and the cow to obtain estimates of the nature of the mammalian mtDNA substitution process over an 80 million year period. We studied the temporal behavior of several characteristics of DNA sequence variation and divergence and compared our model's prediction with the observed estimates. Our results are consistent with strong underlying mutational biases. In addition, our results confirm an extremely rapid rate of nucleotide substitution for mammalian mtDNA. Only a portion of the molecule evolves at this high rate, however. Our calculations suggest that approximately one-half of the mtDNA molecule is under severe selective constraints. The nature of these constraints appears to be diverse but is clearly related to conservation of amino acid sequence in protein coding regions and to secondary structure conservation in transfer RNA genes.

Comparison of available Drosophila mtDNA sequence data with predictions of our model indicates that the strong bias favoring transition substitutions is not general to all animal mtDNA and supports our hypothesis that the mammalian mtDNA substitution bias may be due to biases inherent in the mtDNA replicating enzyme.

PLANS FOR SUBSEQUENT YEARS: Our efforts on this project have ended. However, efforts to characterize what mutational pathway biases, if any, exist for the mtDNA replicating polymerase (Pol-γ) are underway in the laboratory of Dr. T. A. Kunkel.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The results of these studies contribute to the knowledge of the extent of naturally occurring sequence variation in human mitochondrial DNA and provide a framework in which to interpret and study the nature of the substitution process in mammalian mitochondrial DNA and the nature of and susceptability to mutagenic damage due to mutagenic agents.

PUBLICATIONS

Aquadro, C.F. and Greenberg, B.D.: Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. Genetics 103: 287-312, 1983.

Aquadro, C.F., Kaplan, N., and Risko, K.J.: An analysis of the dynamics of mammalian mitochondrial DNA sequence evolution. Molecular Biology and Evolution 1: (in press September issue), 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61027-02 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transmission of Cryptic Mutations in Destabilized X Chromosomes of Drosophila

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. H. Judd	Research Geneticist	LG, NIEHS
	J. W. Jack	Senior Staff Fellow	LG, NIEHS

COOPERATING UNITS *(if any)*

Dr. Johng K. Lim, Professor of Biology
University of Wisconsin, Eau Claire

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

A stable Drosophila X chromosome can be destabilized by association for only one generation with the unstable X chromosome (Uc) or its derivatives. Occasionally, male flies carrying the destabilized X chromosome transmit a mutant allele not apparent in their phenotype (cryptic mutation). We are interested in learning about the origin and the underlying molecular mechanisms for transmission of cryptic mutations.

In our system, two X-linked loci exhibit a high frequency of generation and transmission of cryptic mutation. These are cut wings (ct) and forked bristles (f) loci. In a particular cross, for example, more than 2% of males carrying the destabilized X chromosomes produce at least one sperm with cryptic ct. Our data show that a transposable element called gypsy is involved in transmission of the mutations. A number of separate lines of evidence indicate that the ordinary meiotic recombination process is not involved in generating the cryptic mutations.

Suppressible insertion mutations, transposon-mediated instability, heteroduplex structure of cryptic mutant loci, or involvement of extrachromosomal replicating elements are some of the models we have been testing.

PROJECT DESCRIPTION

PROBLEM: Systems of highly mutable genes in both eukaryotes and prokaryotes have been known for decades. The characterization of the transposable DNA sequences responsible for the unusually high mutation rates is moving forward rapidly as these elements are cloned and analyzed in molecular detail. It is still not clear, however, what the conditions are that stimulate the mobilization of particular types of transposons. Particularly interesting is understanding how retrovirus-like sequences may become activated since these are widely distributed in higher organisms, particularly mammals including humans.

OBJECTIVES: We are striving to understand the nature and function of the retrovirus-like transposon gypsy in Drosophila melanogaster which can be mobilized to a high degree in individuals of particular genotypes. We want to understand the mechanism of and the conditions for the mobilization process.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: J. Lim has described the genetical and cytological properties of a highly mutable system in Drosophila melanogaster. We have approached the understanding of this important phenomenon by isolating and characterizing the transposable element that is responsible for the high mutation rates. We are attempting to describe fully the behavior of the element, the conditions under which it is activated and the nature of the mutational changes it imposes on genes where it inserts and/or excises.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: We recently have determined that the mutational instabilities described by Lim are associated with the mobilization of the retrovirus-like element, gypsy. The instability seen in Lim's crosses is resident in the strain carrying the recessive markers y^{59} z^1 w^1 c^6 f. Probing the polytene chromosomes of this strain in situ with labeled gypsy DNA shows 35-60 copies of gypsy in euchromatic sites and an undetermined number in chromocentral heterochromatin. Crossing either males or females of this strain to individuals from a strain marked with m (which has no gypsy sequences in euchromatin and only a few in heterochromatin) produces hybrids that in turn produce a high frequency of cut locus lethals and visible mutations (0.4-0.5%) among their offspring. Analysis by in situ hybridization and Southern blot analysis shows that essentially all such mutations are associated with the insertion of a copy of gypsy into the cut locus. High mutation rates (1.6-5.9%) are also found when F_2 males are tested for the generation of sex-linked lethals. Most mutational events occurring in F_1 or F_2 are clusters, indicating pre-meiotic origins. No P factor sequences are found in either of these strains.

An unusual facet of the mutational process was discovered when m F_2 sons from the matings: y^{59b} z^1 w^i ct^6 f ♂ x m/m ♀

y^{59b} z^1 w^i ct^6 f/m ♀ x m ♂

are mated to In(1)dl-49, y ct^{ns} f g f ♀. About 1 in 100 such matings produce ct mutant offspring, about half of which appear as clusters of ct and ct^+.

These seem to be due to insertion of gypsy into one of several sites in the cut locus of the m chromosome. In those cases where the gonad is entirely ct, however, the cut mutants are identical to ct^6 from the unstable chromosome. Not only do they contain gypsy inserted into the same restriction fragment as the original ct^6 allele, but they also possess an EcoRI restriction site about 5kb proximal to the gypsy insert that is not found in the cut locus from the m chromosome. It is important to note, however, that the F_2 male through which this mutant locus was transmitted was ct^+ m in phenotype.

We are attempting to determine the mechanism for this cryptic transmission of all or part of the cut locus. A possible explanation is that F_2 males may occasionally carry two copies of all or a part of the cut locus. Because the transmission is non-Mendelian, one copy might exist as an extrachromosomal element generated in association with the mobilization and imprecise excision of gypsy.

We are also trying to define more precisely the conditions under which gypsy is mobilized. Of particular interest is determining whether it is stimulated to move by the presence of other transposable elements or by environmental stress imposed by any of several agents.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Cryptic mutations arising through homologue destabilization violate at least two basic tenets of genetics: the stability of genes and the structural integrity of alleles in hybrids. If a gene is so unstable that one cannot predict gametic genotype of an individual with a defined phenotype, Mendelian principles are utterly useless. Somewhat related to gene stability is structural integrity of genes in hybrids. Alleles in hybrids are supposed to maintain structural integrity and this is one of the fundamental assumptions necessary for predicting the genetic ratios. Homologue destabilization clearly show that one chromosome affects the integrity of its homologue which in turn leads to genetic instability.

Study of genetic instability is as old as the rediscovery of Mendel's principles. Yet until recently, the phenomenon of genetic instability has been treated as rare exceptional events. Presence in all genomes of substantial amount of repetitive sequences and wide occurrence of transposable elements discovered through recombinant DNA techniques argue otherwise. It is important to understand the destabilization process and to determine whether it is a general phenomenon that may under some conditions occur in all eukaryotic organisms including humans.

PUBLICATIONS

Lim, J.K., Simmons, M.J., Raymond, J.D., Cox, N.M., Doll, R.F., and Culbert, T.P.: Homologue destabilization by a putative transposable element in Drosophila melanogaster. Proc. Nat'l. Acad. Sci. 80: 6624-6627, 1983.

Lim, J.K., and Crowe, D.J.: The origin of the destabilized Drosophila X chromosome is in the germ cells of certain hybrid females. Genetics 107: S63 (Abstract), 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61028-02 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mutations Affecting the Expression of an RNA Polymerase II Locus in Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lillie L. Searles	Staff Fellow	LG, NIEHS
Others:	Robert A. Voelker	Research Geneticist	LG, NIEHS
	Mary L. Tate	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to characterize mutational events associated with insertion and excision of the transposon P element at the RNA polymerase II locus, RpII^{215}, of Drosophila. Several P element insertion mutations which affect the expression of this gene have been analyzed at the molecular level. The insertions are clustered in two small subintervals of the locus, one region within coding sequences and the other region near the 5' end of the gene. Detailed analyses have focused on two of these insertions, D50 and W42, both of which lie near the 5' end of RpII^{215} and reversions of D50 which restore variable amounts of activity to the gene.

Wild type DNA in the 5' region of the RpII^{215} gene has been sequenced. Within this region the sites of insertion of mutants D50 and W42 have been localized by sequencing. Although both insertions lie within the same region, the precise site of W42 insertion is 80 base pairs upstream of the D50 insertion site.

DNAs from four D50 revertants have been cloned and sequenced; these were shown to be the result of either partial or complete loss of P element. Of the partial reversions two mutants, whose deletion breakpoints differ only slightly from each other, retain a portion of the inverted repeat sequences from the ends of P element. Another revertant was generated by internal deletion but retains almost 400 base pairs of P element DNA. The level of activity in these revertants seems to be determined by the nature of P element sequences that remain rather than the amount of P element DNA.

PROJECT DESCRIPTION

OBJECTIVE: The purpose of this study is to characterize mutational events associated with insertion and excision of the transposon P element at the RNA polymerase II locus, $RpII^{215}$ of Drosophila.

MAJOR FINDINGS AND PROPOSED COURSE: A number of P element insertion mutations which affect the expression of $RpII^{215}$ have been analyzed at the molecular level. The locus was initially cloned from the P element insertion mutant D50 by "transposon tagging." Earlier studies revealed that P insertions at $RpII^{215}$ were clustered in two small subintervals of the locus, one region within coding sequences and the other region near the 5' end of the gene, possibly outside of coding sequences. Detailed analyses have focused on two of these insertions, D50 and W42, both of which lie near the 5' end of $RpII^{215}$ and reversions of D50 which restore variable amounts of activity to the gene.

Approximately one kilobase pairs of wild type DNA in the 5' region of the $RpII^{215}$ gene have been sequenced. Within this region the sites of insertion of mutants D50 and W42 have been localized by sequencing. Although the sites of insertion of these two mutants lie within the same small restriction fragment, the precise sites are different with the W42 insertion site located 80 base pairs upstream of the D50 site. Thus, there are at least three sites of P element insertion at $RpII^{215}$. Results from these studies and studies performed at the white locus suggest that P elements do not insert randomly but rather have preferred sites for insertion. Indeed, the DNA sequences at the D50 and W42 insertion sites are homologous to the consensus sequence for a P insertion site. It is likely that both insertions lie within DNA sequences that are normally transcribed at $RpII^{215}$, but they probably lie outside of coding sequences since there are no open reading frames in this region.

Cloned DNAs from four D50 revertants as well as the entire D50 P element have been sequenced. One of these revertants completely and precisely lost P element and thus is identical to wild type. The other three revertants are the result of partial deletions of P element. Two of these revertants retain 35 base pairs of P element DNA but differ from each other in that they have slightly different deletion breakpoints. The P sequences that remain are a portion of the inverted repeat sequences from the ends of the P element. The other revertant sequenced was also generated by internal deletion but retained 390 base pairs of P element DNA. Thus we have shown that P element can excise both precisely and imprecisely. The imprecise excisions may be generated by the same mechanism that gives rise to the variety of P elements (including D50), which are derived from the larger P factor by internal deletion.

The amount of gene activity (determined in a genetic assay of viability) is variable in the different revertants, and a few generalizations can be made. While precise revertants have normal levels of activity, the partial revertants have reduced activity. The level of activity in the partial revertants seems to be determined by the specific P element sequences that remain rather than the amount of P element DNA. For example, the two revertants which have only 35 base pairs of P element DNA have lower activity than the revertant with 390 base

pairs of P element. Since the 35 base pairs comprise an inverted repeat, perhaps secondary structures would form which might interfere with transcription or translation, resulting in lower activity.

These studies have been completed and are being written for publication.

SIGNIFICANCE AND BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In mammals, transposon-like elements have been implicated in the alteration of gene function resulting in the development of cancer in some cases and perhaps other disorders as well. Using Drosophila transposons as model systems, we may gain a better understanding of how mobile genetic elements affect gene expression.

PUBLICATIONS

Voelker, R.A., Greenleaf, A.L., Gyurkonics, H., Wisely, G.B., Huang, S.-M., and Searles, L.L.: Frequent Imprecise Excision Among Reversions of a P Element-Caused Lethal Mutation in Drosophila. Genetics 107: 279-294, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61029-02 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cloning and Characterization of the Vermilion Locus of Drosophila

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lillie L. Searles	Staff Fellow	LG, NIEHS
Others:	Robert A. Voelker	Research Geneticist	LG, NIEHS
	Mary L. Tate	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of this project are to clone the vermilion locus of Drosophila, to study the structure and expression of the gene, and to investigate the nature of vermilion mutations that can be suppressed by mutating the suppressor of sable locus.

Vermilion encodes the enzyme tryptophan oxygenase which catalyzes the first step in the synthesis of the brown eye pigment. DNA from this locus was cloned from a mutant containing a P element insertion at vermilion by "transposon tagging." The cloned DNA hybridizes to the polytene chromosome band that contains vermilion. Furthermore, DNAs from a number of vermilion mutants show detectable aberrations in the region homologous to the cloned DNA.

Several spontaneous mutations at vermilion, sable, purple, and speck are suppressible by mutations at suppressor of sable. Using cloned vermilion DNA probes, we have determined that these suppressible vermilion alleles are DNA insertion mutations, and DNAs from several of these mutants have been cloned. The mutants v^1, v^2 and v^k are insertions of the copia-like element known as 412. The weakly suppressible mutant v^{36f} contains an insertion of the element known as roo or B104. Work is continuing to determine the manner in which these insertions disrupt gene expression.

PROJECT DESCRIPTION

RESEARCH PROJECT:

PROBLEM: The vermilion locus of Drosophila encodes the enzyme tryptophan oxygenase which catalyzes the first step in the synthesis of the brown eye pigment. This gene, located on the X-chromosome at the cytological band 10A1,2, is of interest for investigation from at least two standpoints. Firstly, measurements of enzyme activity indicate that the expression of tryptophan oxygenase occurs at specific stages of development and that a large proportion of the activity is localized in the head of the fly.

Secondly, several spontaneous mutations at vermilion, purple, sable, and speck are suppressible by mutations at an unlinked locus known as suppressor of sable. In analogous systems, such as suppressor of Hairy wing and suppressor of white-apricot, it has been shown that the mutations which can be suppressed are insertions of specific transposable elements. For example, suppressor of Hairy wing alleviates the effect of some insertions of the mobile element gypsy while suppressor of white-apricot suppresses a mutation caused by insertion of the copia element. In each system it appears that inactivation of the normal product from the suppressor locus results in suppression, suggesting that the active suppressor gene product might interact with specific elements to cause the mutant phenotype. However, the mechanism of suppression is not understood in any of these systems. Since vermilion is a relatively simple, well-defined locus, it should prove useful for studying the interaction of the suppressor of sable locus with vermilion.

OBJECTIVES: We intend to investigate the molecular basis of the developmentally specific expression of tryptophan oxygenase. Furthermore, we will investigate the nature of mutations at vermilion that are suppressible, determine how the mutations disrupt gene expression and how these effects are relieved.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The strategy employed is to clone the vermilion locus, to use cloned DNA as hybridization probes to determine which sequences comprise the vermilion transcript and during which stages of development transcription occurs. Cloned vermilion DNA will also be used to probe DNA from the mutants, to localize the mutations relative to the gene and to determine what types of changes have occurred in the mutants. If the mutations are insertions, then the cloned element(s) can be used to determine by in situ hybridization if the same mobile element is associated with suppressible alleles at purple, sable and speck.

We will determine if the vermilion transcript is altered in the mutants: Is it reduced in amount? If the transcript is still produced, does it have the same initiation or termination points? Then we will ask if suppressor of sable alters transcription of the vermilion mutants. These studies will provide the basis for more detailed investigations of the mechanism(s) regulating vermilion gene expression and the mechanism of suppression.

An understanding of how the expression of a eukaryotic gene is regulated, as well as an understanding of the effects of transposable elements on gene expression and how these effects are suppressed could make a significant contribution toward resolving problems relating to some genetic disorders in higher organisms.

RECENT ACCOMPLISHMENTS: We have succeeded in cloning DNA from the vermilion region. A genomic library was made from a vermilion mutant containing at least 40 P elements, one of which was within the polytene chromosome band (10A1,2) that contains vermilion. In the first collection of 26 P element-containing clones that were purified, one clone hybridized to 10A1,2. DNAs from a number of vermilion mutants show detectable aberrations in the region homologous to the cloned DNA. Subsequently, we have cloned DNA from wild type flies spanning approximately 30 kb in the vermilion region.

From genomic Southern analysis we have determined that all of the suppressible vermilion alleles, indeed, contain insertions. Three of these spontaneous mutants v^1, v^2 and v^k contain the same insertion, i.e., the insertion mutants have the same restriction pattern. A fourth mutant v^{36f}, which is only weakly suppressible, contains an insertion in an adjacent restriction fragment, the same fragment which contains the P element insertion.

Vermilion DNA from the mutants v^2, v^k and v^{36f} has been cloned. The insertion in v^k and v^2 is the 7 kb "copia-like" element known as 412. The v^{36f} insertion the 9 kb element known as roo or B104. (It is not clear whether v^{36f} is poorly suppressed because the element is different or because the position of the insertion is different). All of these insertions, 412, roo and P, lie in close proximity to each other within a 1 kb region of DNA.

In situ hybridization studies are in progress, and we have thus far determined that the suppressible speck and purple mutants contain 412 insertions at the cytogological bands which contain these loci. This observation supports the idea that suppressor of sable suppresses some insertions of the 412 element. However, since there are on the average about 40 copies of the 412 element per genome, it is possible that the association of the element with the chromosome bands containing speck and purple is fortuitous. Further studies may strengthen the validity of this result.

Thus, all of the mutations analyzed to date that are suppressible are insertions of some type of "copia-like" element, all of which have a structure similar to retroviruses. One property of "copia-like" elements is that they are actively transcribed at specific stages. Perhaps the initiation or termination of transcription within these elements interferes with transcription at vermilion and other affected loci. In fact, results of studies on suppressor of white-apricot indicate that the suppressor affects termination of white locus transcription within the copia element. It is conceivable that some suppressors might affect transcription initiation within the element.

PLANS FOR SUBSEQUENT YEAR: Transcription studies at vermilion will be a major focus for the coming year. First we need to identify which sequences are transcribed, to localize the 5' and 3' ends of the transcript as well as to localize introns if they exist. From these studies we can determine if the insertions lie within the structural gene, in introns, or outside the gene. We will analyze mutant transcripts in the presence and absence of suppressor of sable to determine if the suppressor affects transcription.

A developmental profile of transcription will also be determined. From measurements of enzyme activity it seems that there are two peaks of tryptophan oxygenase activity, one during the pupal stage and another in newly eclosed adults. It will be interesting to see if the pattern of transcription activity parallels enzyme activity and if the transcripts produced during the two stages differ in any respect.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61030-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular analysis of the Om mutator in Drosophila ananassae

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. H. Langley	Research Geneticist	LG, NIEHS
Others:	Antony E. Shrimpton	Visiting Fellow	LG, NIEHS
	Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hinton (1984) described an unusual mutator in D. ananassae which he speculated was a transposable element with previously unknown properties; namely the element could only be phenotypically detected by its effect on eye morphology at 15 non-pleiotropic, non-dosage compensated, non-random loci. This element was known to originate from an X chromosome in a particular stock, ca;px. With the recovery of a spontaneous singed mutation, sn^{9g}, in an ocullar morphology (Om) mutant line derivative, Om(1D)9g, it was possible to investigate his speculation. D. melanogaster singed, sn, DNA probes were used to isolate and recover a D. ananassae singed gene. A comparison of sn^{9g} and wild type singed restriction map implicated a 6½ kb insert as the element responsible for the mutator effect. This insert was shown by Southern blot and in situ hybridization to be repetitive and dispersed.

A total of 186 recombinant lines from four X-linked Om loci were examined. 80 were Om and 106 were non-Om; in all instances an in situ hybridization signal, when probed with sn^{9g} insert, was found at appropriate locations on the polytene chromosomes. Linkage was complete and showed that the sn^{9g} insert was homologous to sequences localized at the sites of Om mutants.

The most likely hypothesis is that the Om element is inserting at a target site encoded in a control sequence found at eye morphology genes. We intend to study further this phenomenon and to check this hypothesis by characterizing the Om element and insertion target sites. Of particular interest is the possibility of elucidating further the nature of eukaryotic gene structure and control, as well as to increase our knowledge of the nature of mobile genetic elements and their role in spontaneous mutation.

PROJECT DESCRIPTION

NATURE AND OBJECTIVES OF PROJECT: Hinton (1984) speculated that Ocullar Morphology (Om) mutability in D. ananassae involved a transposable element whose target site of insertion was encoded in the control sequence found at eye morphology genes. Om mutability requires homozygosity of the ca;px stock X chromosome, or, in heterozygous females, the presence of either of two self-replicating extrachromosomal elements involved in male crossing over or mutability. Om mutability involves the spontaneous mutation at approximately 15 non-random loci, resulting in semidominant, non-dosage compensated, non-pleiotropic eye morphology mutations. While alleles of the same locus have similarities, they could not be unambiguously assigned to loci by phenotype alone. Om(1D)9, a particularly unstable allele, produced several derivatives, one of which, Om(1D)9g, was more extreme and was accompanied by a linked singed mutant, sn^{9g}.

The purpose of the present study was to establish the connection between Om mutability and sn^{9g}, i.e., to investigate whether sn^{9g} is the result of a mistaken Om event. The singed allele was chosen as it was from one of the very few loci mutated in Hinton's Om experiments that had been cloned in Drosophila.

METHODS EMPLOYED: The Cloning of the Om element. DNA probes from the singed locus of D. melanogaster were used to clone the homologous region of D. ananassae. A comparison of sn^{9g} and wild type singed loci detected a 6½ kb insert in the sn^{9g} locus. Genomic Southern blotting and in situ hybridization techniques were used to show that this insert was repetitive and dispersed. There were a preponderance of copies on the X chromosome and a survey of several stocks indicated that this element was absent from some stocks.

Recombinant Om lines from 4 Om stocks were manufactured, isolated and made homozygous for in situ hybridization with the 6½ kb insert clone. Complete genetic linkage between the Om phenotype and in situ hybridization signal was demonstrated.

MAJOR FINDINGS AND PROPOSED COURSE: The 6½ kb insert cloned from sn^{9g} was shown to be homologous to a sequence found at Om mutations in D. ananassae. With the exception of the sn^{9g} mutation the Om mutator is unique in that it is phenotypically detected through its affect on eye morphology at a fixed number of sites. The next step in this project is to clone and characterize several Om insertions and their flanking regions to determine the underlying mechanisms. We will attempt to characterize the Om mutant derivatives and determine whether it is the element, the site of insertion, or an interaction between the two that is responsible for the eye morphology effect. To determine the physical structure and properties of the element and to determine the target site for insertion, is a necessary part of the investigation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In a comparatively short time the generally accepted view of a largely rigid immobile genome has been changed by the discovery and study of mobile genetic elements. The role of these elements in mutation, cancer, evolution and speciation is of

great interest and importance; therefore, a delineation of their properties is essential. Some of the hypothesized properties of the Om element are unprecidented since the phenotypic effects of the element are only detected at 15 or so non-random sites and only have an effect on eye morphology; also, the Om element is X-linked.

The most likely model of Om mutability involves a transposable element whose target site of insertion is encoded in the control sequence of eye morphology genes (Britten and Davidson, 1969). Clearly if this is so then the study of Om mutability will further our understanding of development and eukaryotic gene structure and control.

The uniqueness of this mutator system may afford some new understanding of the biology of transposable elements and their impact on host organisms. Basic knowledge of this potential source of spontaneous mutation is fundamental to the understanding of genetic disease.

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 61031-01 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Molecular Population Genetics of Transposable Elements

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C.H. Langley	Research Geneticist	LG, NIEHS
Others:	Elizabeth A. Montgomery	Bio. Lab. Tech.	LG, NIEHS
	Clara S. Millis	Guest Worker	LG, NIEHS
	Shiu L. Huang	Guest Worker	LG, NIEHS

COOPERATING UNITS *(if any)*

Dr. Shiu L. Huang and Clara S. Millis
Environment Health Research and Testing Inc.
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LAB/BRANCH

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	0.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The types of gene structural changes causing deficiency of hypoxanthine guanine phosphoribosyl transferase (HGPRT) activity in spontaneous mutations is being examined in cultured human fibroblasts. The deficiency of this enxyme activity causes a human disease (Lesch-Nyhan Syndrome). The restriction enzyme cleavage patterns of HPRT gene sequences in mutant lines will be analyzed. The work is presently focused on obtaining a large number of independent spontaneous mutants that existed in new born baby foreskins. Ten independent mutants have been isolated from different human subjects. The mutant cells were grown to large numbers. Portions of cultured cells were frozen in liquid nitrogen for cytogenetic and enzymology studies at a future time; while portions of cultured cells were frozen for DNA extraction. Southern blot analysis is now in progress to assess the possible involvement of DNA rearrangements in spontaneous mutation.

PROJECT DESCRIPTION

PROBLEM: It has been well established that deficiencies of the protein products of hypoxanthine-guanine phosphoribosyl transferase cause a human genetic disease (Lesch-Nyhan syndrome). However, little is known about the nature of the genetic changes that give rise to variations in phenotype.

OBJECTIVES: The objective of this project is to look for the structural changes in the DNA of the spontaneous mutations of the HPRT gene in human tissues and relate the changes to and understand the cause of the human genetic disease.

SCIENTIFIC JUSTIFICATION AND EXPERIMENTAL APPROACH: The recent cloning of the human HPRT structural gene sequences has now made it possible to carry out the structural analysis of mutant human HPRT genes. Identification of mutation in cultured cells and fetuses previously had been limited to enzyme assays. The enzyme activity of mutants ranges from none to full activity. It is important to understand the relationship between the residual enzyme activity of the gene products and the structure of genes, at the molecular level. Primary human cell cultures are established from neonatal foreskins. To assure the independent origin of mutants, one mutant from each primary culture is used for molecular analysis. Mutant cells form colonies in 6-TG medium. Through several culture passages, the mutant cells grow to large numbers. To determine the nature of mutations at the molecular level, DNA is purified from each of the mutants and is digested with restriction endonucleases. After fractionation by agarose gel electrophoresis, HPRT-containing sequences can be visualized by Southern blotting and hybridization with a non-repetitive fragment of the HPRT clone labeled to high specific activity by nick-translation.

RECENT ACCOMPLISHMENTS: Since normal human cells senesce, mutant cells derived from non-primary culture cease to grow before a number of cells sufficient for analysis can be obtained. Primary cultures, therefore, offer several advantages. 1. Mutants cells are young and can yield enough DNA for analyses. 2. Mutants isolated from foreskins are preexisting ones and represent mutations in vivo. 3. Mutants from different individual are of independent origin. Fifty foreskins have been collected, from which primary cultures have been established. Mutants from 15 primary cultures have been isolated and stored in liquid nitrogen. Ten mutant cell lines have been grown to 5-6 x 10^6 cells for DNA extraction.

PLANS FOR SUBSEQUENT YEAR: Work will continue to isolate mutants from each primary culture. We are presently in the process of isolating and purifying DNA from mutant cell lines, and refining molecular techniques for human DNA analysis. Experiments will be carried out in next two years to analyze the genetic structure of mutant DNA and to measure the residual enzyme activity of mutants.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The nature of spontaneous mutations is an important component of a fundamental understanding of genetic disease. Insertions are a major part of spontaneous mutation in several experimental organisms. It is important to determine whether this is also the case in human cells (both germline and somatic).

PUBLICATIONS

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61032-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure-function of mammalian lactate dehydrogenase isozymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Steven S.-L. Li	Research Geneticist	LG, NIEHS
Others:	Farida S. Sharief	Biologist	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The complete primary structure of human LDH-A4 isozyme has been determined by sequence analyses of LDH-A cDNA and protein. Amino acid sequence analysis of mouse LDH-A4 isozymes had positively identified 80% of the 331 residues, and the remaining amino acids were aligned on the basis of peptide compositions and homology with other known LDH sequences. Partial amino acid sequences of LDH-B4 isozymes from human and mouse have also been determined by amino acid sequencing. The amino acid sequences of 100% of the 330 residues from mouse testicular LDH-C_4 and 84% of rate LDH-C_4 have been determined. Sequence comparison among mammalian LDH isozymes clearly indicates that A_4 (muscle) and B_4 (heart) isozymes shows a closer evolutionary relationship to each other than either to the C_4 (testis) isozyme. Recently, LDH-A_4 isozymes have been found to be single-stranded DNA binding proteins which may play important roles in DNA replication, repair and recombination. The relationship of the protein structure to multiple functions as enzyme and as DNA binding protein will be further studied by *in vitro* directed mutagenesis.

PROJECT DESCRIPTION

PROBLEM: Structure and function of mammalian ssDNA-binding lactate dehydrogenase proteins.

OBJECTIVES: In mammals and birds three homotetrameric LDH isozymes A_4 (muscle), B_4 (heart) and C_4 (testis) possess distinct physical, catalytic and immunological properties. Recently, the ssDNA-binding protein from rat liver was found to be extremely homologous to pig LDH-A sequence. The structural relationship of the mammalian LDH proteins will be correlated with the ssDNA-binding and enzymatic activities.

EXPERIMENTAL APPROACH AND SCIENTIFIC JUSTIFICATION: The primary and 3-D structures of mouse and rat LDH-C_4 have been determined in my laboratory at NIEHS. The 3-D structure of dogfish LDH-A_4, pig LDH-B_4 and mouse LDH-C_4, and amino acid sequences of dogfish and pig LDH-A_4 and pig LDH-B_4 were reported by other laboratories. We are now determining the covalent structure of LDH-A_4 and B_4 isozymes from human and mouse by the direct protein sequencing and/or deduced from the nucleotide sequences of cDNA and genomic clones. The structural relationship of the LDH proteins to the ssDNA-binding and enzymatic activities will be investigated by the *in vitro* directed mutagenesis and post-translational modification.

In human, the different clinical features of LDH-A or LDH-B deficiencies have been correlated with the impaired ATP production under anaerobic condition for LDH-A, but not LDH-B deficiency. The LDH-A_4 isozyme has also been shown to increase predominantly in many human cancer tissues. The LDH-A_4 isozyme along with enolase and phosphoglycerate mutase have been reported to be phosphorylated at tyrosine residue(s) in cells transformed by Rous sarcoma virus. The functional significance of these structural modifications will be investigated. A new LDH-K isozyme has been found in many carcinomas and in cultured cells transformed by the Kirsten murine sarcoma virus and it may also be expressed in normal retina. The structural relationship of the LDH-K isozyme to the known LDH-A_4, B_4 and C_4 isozymes will be determined.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: The primary structure of human and mouse LDH-A_4 isozyme has been determined by sequence analyses of the protein and cloned cDNA. Partial amino acid sequences of human and mouse LDH-B_4 have also been obtained by direct protein sequencing. The structures of LDH-C_4 isozymes from mouse and rat testes have been established by amino acid sequencing and the space-filling models of the LDH-C_4 molecules have been constructed on a computer-graphic system.

This available information on primary and 3-D structures of mammalian LDH-A_4, B_4 and C_4 isozymes makes it possible to discuss their functional, immunological and evolutionary relationships. The different amino acid sequences at the LDH active center have been correlated with different kinetic properties of LDH-A_4, B_4 and C_4 isozymes. The amino acid sequence of the loop region of sperm-specific LDH-C_4 was shown to be quite different from those of somatic LDH-A_4 and

B_4 isozymes, and it may account for its unique physical and chemical properties: thermal stability, broad substrate specificity and low turnover numbers. The immunological properties of mouse and rat LDH-C_4 have been compared with those of mouse LDH-A_4 and LDH-B_4 isozymes, and the potential antigenic determinants of LDH-C_4 isozymes have also been predicted on the basis of the hydrophilicity analysis of their amino acid sequences and three-dimentional protein structure. The LDH-C_4 isozyme is immunologically distinct from LDH-A_4 and LDH-B_4 isozymes, and the active immunization of animals with LDH-C_4 autoantigen has lead to immunogenic impairment of fertility in females. Amino acid sequence comparison among vertebrate LDH isozymes clearly indicates that A_4 (muscle) and B_4 (heart) isozymes show a closer evolutionary relationship to each other than either to the C_4 (testis) isozyme.

FUTURE RESEARCH PLANS: 1. The primary structure of human and mouse LDH-B, and human LDH-C proteins will also be determined by sequence analyses of the proteins and cDNAs. The potential antigenic determinants of human LDH-C_4 isozyme will be analyzed and some of antigenic peptides may be synthesized chemically and tested for possible impairment of fertility.

2. The in vitro mutagenesis of LDH genes will be used to illustrate the structural relationships to ssDNA-binding and enzymatic activities of LDH proteins. The molecular nature of spontaneous and induced mutant LDH-A_4 proteins from mouse may also be determined.

3. The structural modification of LDH isozyme by oncogene src tyrosine kinase and its functional significance will be investigated. The dephosphorylation of tyrosine residue(s) by acid phosphatase will also be studied. The possible relationship between the LDH isozymes and oncogene ras proteins (H-ras, K-ras & N-ras) will be investigated.

PUBLICATIONS

Pan, Y.-C.E., Sharief, F.S., Okabe, M., Huang, S. and Li, S.S.-L.: Amino acid sequence studies on lactate dehydrogenase C_4 isozymes from mouse and rat testes. J. Biol. Chem. 258: 7005-7016, 1983.

Li, S.S.-L., Feldmann, R.J., Okabe, M., and Pan, Y.-C.E.: Molecular features and immunological properties of lactate dehydrogenase C_4 isozymes from mouse and rat testes. J. Biol. Chem. 258: 7017-7028, 1983.

Li, S.S.-L., Fitch, W.M., Pan, Y.-C.E., and Sharief, F.S.: Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A_4 (muscle), B_4 (heart) and C_4 (testes). J. Biol. Chem. 258: 7029-7032, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 61033-01 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Analysis of Genetic Variation in lens crystallin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Loren C. Skow	Sen. Staff Fellow	LG, NIEHS
Others:	Maria K. Donner	Fogarty Fellow	LG, NIEHS
	Shu-Mei Huang	Research Associate	LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Genetics

SECTION
Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To facilitate our studies of mouse eye mutants, we have been searching among inbred strains for genetic variation in lens crystallin genes using cDNA probes for α-, β-and γ- crystallins. Southern blot analysis of α-crystallin sequences has detected three restriction fragment size classes (Acry-1^{a,b,c}) among 42 inbred strains of mice. The polymorphisms are due to insertions flanking the structural gene. Gene mapping experiments using 69 recombinant inbred strains demonstrated close linkage of Acry-1 to H-2K on chromosome 17. Estimated recombination distance is 1.4 cM. The Acry-1 -H-2 linkage was confirmed by analysis of 18 H-2 congenic and recombinant congenic strains, in which the presence of donor Acry-1 was completely concordant with the presence of donor H-2K. The Acry-1 - H-2 association has been maintained during the development of inbred strains and suggest that strong linkage disequilibrium may exist for these genes in natural populations. In only 1 of 42 strains tested was the H-2 haplotype not predictive of Acry-1 alleles. Conservation of genes in the MHC region of mice and humans implies that the human α-crystallin gene is located on chromosome 6.

Analysis of β-crystallin sequences (Bcry) by 17 endonucleases has failed to detect restriction polymorphisms in DNA from representative strains of mice.

In contrast, γ-crystallin sequences (Gcry) have demonstrated restriction polymorphisms with all eight endonucleases so far employed. Genetic mapping experiments have been conducted on the γ-crystallin sequences using 48 recombinant inbred strains. We have found no recombination between Gcry and a γ-crystallin electrophoretic variant (LEN-1) previously mapped to chromosome 1 very close to the mutant gene, Elo, which produces anophthalmia in mice.

PROJECT DESCRIPTION

PROBLEM: The vertebrate eye is exquisitely sensitive to chemical and physical damage and is widely used as a test organ in genetic and somatic toxicology. Little is known about the normal development and biochemistry of the eye. Consequently it is difficult to assess the mechanisms involved in ocular toxicology. Numerous non-allelic mutations affecting eye development and function are maintained in mice and many of these mutations have been induced by chemical or physical mutagens. The purpose of this project is to use induced and naturally occurring genetic variation to investigate the processes controlling development and function of the eye.

OBJECTIVES:

1. Locate and determine the structural features of the α-, β- and γ-crystallin electrophoretic variants and restriction fragment polymorphisms in or near the genes encoding the crystallin proteins.

2. Conduct genetic and molecular analyses of mouse eye mutants with emphasis on complementation analysis and linkage studies.

3. Evaluate crystallin gene expression in eye mutants with emphasis on expression of γ-crystallin mRNA in the anophthalmic C3H/HeJ - Elo mouse.

ACCOMPLISHMENTS

1. The mouse α-crystallin gene (Acry-1) has been mapped to chromosome 17 by restriction fragment size polymorphisms. Analysis of recombinant inbred and congenic strains have located the gene between the glyoxalase (Glo-1) locus and H-2K about 1.4 cM from H-2K.

2. Analysis of the Acry-1 variation by several restriction endonucleases revealed that the variation is due to insertions in the 3' flanking region. Single fragments of 9.0, 10.5 and 12.0 kbp after Hinc II digestion suggest an insertion element of 1.5 kbp in unit size.

3. Genetic analyses of a spontaneous dominant cataract, NM and a polymorphic locus encoding an eye-specific form of aldehyde (retinal) dehydrogenase (AHD-4) have demonstrated that both variants are inherited as single Mendelian traits. Linkage studies of backcross progeny have not yet assigned NM or ADH-4 to a chromosomal region.

4. Restriction endonuclease analysis of DNA from C3H/HeJ - Elo, an anophthalmic strain and the coisogenic normal strain C3H/HeJ has not detected any structural differences in the γ-crystallin gene sequences of Elo. These results suggest that Elo mice may be abnormal for γ-crystallin gene expression but that the structural genes remain essentially intact.

5. Five crystallin fractions have been purified from mouse lens extracts and used to produce antisera in rabbits. Western blot analysis has revealed that LEN-2, a low molecular weight crystallin of the classical γ-crystallin component, shares antigenic determinants with β-crystallin, thereby confirming the conclusion, based on amino acid composition, that LEN-2 is a member of the β-crystallin family.

6. We have isolated a 12 kb fragment containing the mouse α-crystallin genomic sequence from a Charon 30 library. This fragment will be analyzed to determine the nature of the genetic variation within and flanking the α-crystallin gene.

7. We have prepared a representative collection of DNA from about 120 strains of mice, 17 strains of rats and from human x mouse and cattle x hamster somatic cell hybrid clones for comparative mapping and restriction analysis of crystallin genes and other sequences among these species. Preliminary results from the human x mouse and cattle x hamster somatic cell hybrid clones are consistent with the conservation of the α-crystallin-MHC region in the mammalian genome.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A knowledge of the mechanisms by which genotoxic substances interrupt normal developmental sequences is crucial to ascertain the risk associated with exposure to mutagenic and teratogenic compounds. The development and function of the vertebrate eye is exquisitely sensitive to perturbation by a variety of physical and chemical agents and the vertebrate eye, therefore, presents an excellent opportunity to investigate normal and aberrant developmental patterns. By using the numerous genetic mutants available in the mouse, we will be able to analyze developmental processes in eye formation and come to an understanding of how mutagens and teratogens disturb normal eye development.

RESEARCH PLANS FOR 1984-1985

1. Determine the level of γ-crystallin mRNA and translation products in lenses from newborn C3H/HeJ-*Elo* mice.

2. Evaluate the comparative genetics of the crystallin genes in cattle, humans and rats using panels of somatic cell hybrid clones and inbred strains of rats.

3. Isolate and characterize the insertion element(s) flanking the α-crystallin gene of mice.

4. Continue linkage experiments to locate NM and AHD-4.

5. Continue the search for genetic markers in the β-crystallin genes.

PUBLICATIONS

Popp, R.A., Bailiff, E.G., Skow, L.C., Johnson, F.M., and Lewis, S.E.: Analysis of a mouse α-globin gene mutation induced by ethylnitrosourea. Genetics 105: 157-167, 1983.

Skow, L.C., Burkhart, B.D., Johnson, F.M., Popp, R.A., Popp, D.M., Goldberg, S.Z., Anderson, W.F., Barnett, L.B., and Lewis, S.E.: A mouse model for β-thalassemia. Cell 34: 1043-1052, 1983.

Skow, L.C., Popp, R.A., and Bailiff, E.G.: A second polymorphic lens crystallin (Len-2) in the mouse. Genetics and biochemical analysis of LEN-1 and LEN-2. Biochemistal Genetics (In Press), 1984.

Antonucci, T.K., von Diemling, O.H., Rosenblum, B.B., Skow, L.C., and Meisler, M.H.: Conserved linkage within a four centimorgan region of mouse chromosome 9 and human chromosome 11. Genetics (In Press), 1984.

Frater-Schröder, M., Prochazka, M., Haller, O., Arvert, F., Porck, H. J., Lundin, L.G., and Skow, L.C., Hilkens, I., and Hilgers, J.: Localization of transcobalamin II (Tcn-2) on chromosome 11: Linkage to waved-2 (wa-2) and the hemoglobin e-chain locus (Hba). Biochemical Genetics (In Press), 1984.

Popp, R.A., Popp, D.M., Johnson, F.M., Skow, L.C., and Lewis, S.C.: Hematology of a murine beta thalassemia. A longitudinal study. Annals N.Y. Acad. Science (In Press) 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 65021-12 LG

PERIOD COVERED

October 1, 1983 through September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Investigation of Germinal Mutation Induction in Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. M. Johnson	Research Geneticist	LG, NIEHS
Others:	L. C. Skow	Senior Staff Fellow	LG, NIEHS
	M. L. Snell	Bio. Lab. Technician	LG, NIEHS
	Marjo Smith	Postdoctoral Fellow	LG, NIEHS
	D. P. Lovell	Statistician	BIBRA
	S. E. Lewis	Senior Geneticist	RTI

COOPERATING UNITS *(if any)*

Research Triangle Institute, Life Sciences Group, Research Triangle Park, N.C.; British Industrial Biological Research Association, Carshalton, Surrey, England

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to detect natural and induced mutations in mice for the purpose of providing understanding of the specific molecular events involved in germinal mutation and the effects of these events on the life, form and function of the mammalian organism. Results are relevant to cases of human exposures to mutagens and the potential for increased risk of genetic disease that may accompany mutagen exposure. The problem is approached by detecting mutations at specific biochemical loci with electrophoretic methods, by conducting characterization studies on the mutant genes and gene products, and by examining the animals for expressed physical abnormalities correlated with mutation rate increases and with specific induced-mutant genotypes. The methods have led to successful identification of more than 20 ethylnitrosourea-induced mutants affecting proteins such as malic enzyme, α hemoglobin and phosphoglucomutase, but there is little evidence to suggest that most of the mutants are detrimental to health-related characteristics. The results raise questions as to the extent hypotheses of human genetic risk based upon increased mutation rates are indicative of elevated probabilities for significant genetic damage. As some mutagens are therapeutically beneficial the possibility of false risk estimates and unjustified restrictions on mutagen exposures raise important medical and legal considerations. Previously published mammalian-genetic data are being reexamined to determine if new interpretations are possible, and laboratory experiments involving the use of other mutagens and more powerful detection techniques are planned for the future.

PROJECT DESCRIPTION

METHODS EMPLOYED: Current perceptions of human genetic risk implicate elevated mutation rates as a source of increased genetic disease in human populations subjected to exposure to mutagenic agents in the environment. Although many test methods provide for the efficient detection of mutations, the mouse affords unique experimental opportunities to analyze efficiently the effects of mutations in a mammal with many established homologies with man. Therefore with mice, an effective means to detect mutations and to provide understanding of their effects in terms relatable with human gene expression is available.

Our work has concentrated upon detecting induced and spontaneous mutations in mice using electrophoretic techniques applied to surgically removed tissue samples. After mutant identification, efforts to characterize the variation are undertaken. Characterization includes inheritance studies, analyses of structural alterations in the DNA and gene products, comparative functional analyses of the mutant and normal gene product, and investigations as to how the alterations may affect the life of the animal. The progeny of both mutagen-treated and control parental animals are used. The principal mutagen we have used is ethylnitrosourea.

Subsets of animals derived from the electrophoretic studies have been used to analyze morphological variation in the skeleton. By comparing control and mutagen treated groups it is possible to determine the extent to which increased variation and increased severity of expression is associated with mutagen exposure. Quantitative measurements and multivariate statistical techniques are used. Having mutation frequencies obtained from the electrophoretic studies, any increased morphologically expressed variation can then be correlated with average induced-mutation rates. Furthermore, if correlation is established, corraboration of induced detrimental effects is available from the results of analyzing the effects of the mutations at specific biochemical loci and further genetic studies of the morphological variation.

JUSTIFICATION: According to published approaches to human genetic risk estimates it has been suggested that variously obtained induced mutation-rate data and certain dominant indicators of genetic damage in mice may provide a suitable basis for achieving useful numerical risk values. Once correlation is established, it has been further suggested that short-term mutagenesis tests may alone be sufficient to estimate genetic risk, obviating the need for more time consuming and expensive investigations with mice. Human exposures to agents determined to have the potential for causing increased risk of genetic damage may need to be controlled by law. Our project with mice provides validation of suggested approaches to human genetic-risk estimation. Proper validation is important because particular mutagen exposures (to X-rays for example) have well established therapeutic aspects. Regulations based upon invalid risk estimates would, thus, introduce potential health hazards to those individuals who would be deprived of beneficial mutagen exposures.

RECENT RESULTS AND SIGNIFICANCE: A large series of electrophoretically recognized ethylnitrosurea-induced mutants have been recovered from the experiments. Several spontaneous mutations, among which there was one that arose in the parental generation, have also been identified. The average mutation rate caused by a single 250 mg/kg dose of ethylnitrosourea has been determined to be 100 times, or more, greater than the spontaneous mutation rate. The evidence for a significant detrimental effect accompanying the mutation rate increase has, however, not been compelling. Most of the induced specific-locus mutations do not show demonstrable deleterious effects even though the majority of the mutants appear to be different from previously recognized natural genetic variants. Additionally, morphologically expressed variation was found not significantly increased by mutagen exposure. Skeletal analyses revealed a few extreme morphologically abnormal individuals, but the frequencies of these variants were not significantly different between treated and control groups. These findings suggest highly elevated mutation rates may sometimes occur in the absence of important adverse genetic expression. The results are unsupportive of proposed approaches to human genetic-risk estimation and regulation based upon mutagenic activity as indicated by mutation rate increases. Our negative results do not exclude possible induced detrimental effects below the detection limit as determined by the size and scope of the present experiments. However, it may prove difficult to find a threshold for detection if one, indeed, exists at all. Thus at an estimated average spontaneous mutation rate of 1×10^{-6} per gene and 50,000 genes per haploid genome, our data indicate that the incidence of new mutants increased from a natural level of 5 mutants per 100 individuals to an average of 5 or more mutants per individual as a result of the mutagen exposure. No significant increased expression of adverse genetic effects have been found in comparison of F_1 animals from ENU-treated parents and controls. Accordingly, other forms of toxic expression (not mutation) caused by compounds like ENU, may be more relevant for testing harmful effects on man than measurements of mutagenic activity. However, the results, based only on one mutagen and one pair of mouse strains, may not apply generally. Therefore, definite general conclusions are not possible without having the results of larger, more comprehensive experiments available for analysis. If future work confirms present findings, a practical implication of the results would be that meaningful alternative approaches to currently used genotoxicity methods, as applied widely to chemicals, complex environmental mixtures and the like, would need to be sought in order to properly protect public environmental health.

PLANS FOR SUBSEQUENT YEAR: We plan to reexamine previously published mouse-mutagenesis data to see if reasonable interpretation, consistent with our experimental results, is possible. The majority of previously published work in the field has involved radiation exposures. Depending on the results of reexamining the existing data, additional, more powerful experiments with radiation, ethylnitrosurea or other mutagens will be designed and proposed for consideration.

PUBLICATIONS

Lovell, D.P., and Johnson, F.M.: Quantitative genetic variation in the skeleton of the mouse. I. Variation between inbred strain. Genetical Research 42: 169-182, 1984.

Lovell, D.P., Totman, P., and Johnson, F.M.: Variation in the shape of the mouse mandible. I. Effect of Age and Sex. Genetical Research, (In Press).

Popp, R.A., Bailiff, E.G., Skow, L.C., Johnson, F.M., and Lewis, S.E.: Analysis of a mouse α-globin gene mutation induced by ethylnitrosourea. Genetics 105: 157-167, 1983.

Lewis, S.E., and Johnson, F.M.: Dominant and recessive effects of electrophoretically detected specific locus mutations. In: Utilization of Mammalian Specific-Locus Studies in Hazard Evolution and Estimation of Genetic Risk, Ed. by F.J. deSerres and W. Sheridan. Plenum, NY pp. 267-278, 1983.

Johnson, F.M., and Lewis, S.E.: The detection of ENU-induced mutants in mice by electrophoresis and the problem of evaluating the mutation rate increase. In: Utilization of Mammalian Specific-Locus Studies in Hazard Evolution and Estimation of Genetic Risk, Ed. by F.J. deSerres and W. Sheridan. Plenun, NY pp. 95-108, 1983.

Skow, L.C., Burkhart, B.A., Johnson, F.M., Popp, R.A., Popp, D.M., Goldberg, S.Z., Anderson, W.F., Barnett, L.B., and Lewis, S.F.: A mouse model for β-thalassemia. Cell 34: 1043-1052, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 65029-03 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of Mouse Lens Mutations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Loren C. Skow Sen. Staff Fellow LG, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Genetics

SECTION

Eukaryotic Gene Structure Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 61033-01 LG entitled "Molecular Analysis of Genetic Variation in lens crystallin".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 65032-02 LG

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Induced Mutations at the MOD-1 and GPI Loci of Mice

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. V. Malling	Research Geneticist	LG, NIEHS
	J. G. Burkhart	Research Chemist	LG, NIEHS

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project Number Z01 ES 65033-01 LG entitled "In Vivo Mammalian Mutagenesis".

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 65033-01 LG

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vivo Mammalian Mutagenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. V. Malling	Research Geneticist	LG, NIEHS
	J. G. Burkhart	Research Chemist	LG, NIEHS

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C.A. Hutchinson III, M.H. Edgell, & S.C. Hardies
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Eukaryotic Gene Structure

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	2	0.3

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project is to study gene mutations and small intrachromosomal changes directly in exposed mammals. We have taken two approaches. 1). Site specific alterations in proteins detected in single cells: Antigenic differences between rat and mouse LDH-C were used to detect mutations in mouse sperm. Initial work indicated that this system had potential for the study of mutagenesis in mammals; later research showed that the apparent mutation frequency varied too much to give reproducibly useful data. The conclusion was reached that although antibodies had the needed sensitivity, interspecies differences may not be appropriate as markers for induced mutations; also, a model mutant cell must exist for a proper test of antibodies. Therefore, use of antibodies are being applied to spontaneous and induced mutants of malic enzyme (MOD-1) in mice. Normal (MOD-1) was purified and antibodies produced; sperm and somatic cells were labeled with this antibody. A mutant form of MOD-1 was purified. The future strategy is to produce and test antibody to the abnormal MOD-1 using cells from normal homozygotes and heterzygotes with the mutant allele. 2). Site specific changes detected at the DNA level: A major problem for detection of genetic damage directly in mammalian DNA is that most genes occur in one, or few copies; there are however, two possibilities. The first is the use of mitochondrial (mt) DNA. Cloned mouse mtDNA has been used for restriction analysis of sperm mtDNA isolated from a single mouse; after additional technical improvements, the mtDNA from treated mice will be examined for mutations. The second is the use of viral DNA transformed into mammalian DNA. Double stranded DNA from ϕX174 am3, cs70 was transformed into mouse L-cells. The DNA was incorporated into several places in tandem arrangements. Using restriction enzymes and ligase it was possible to transfect spheroplasts with ϕXDNA from the transformed mammalian cells. Attempts are being made to create a mouse strain with ϕXDNA in the genome for the study of mutation induction in any part of the animal tissue.

PROJECT DESCRIPTION

OBJECTIVES: The primary objective of this project is to understand the mechanisms of mutations, the differential sensitivity of various loci to mutagenesis, the relationship of mutations to the organization of the genome, and the phenotypical consequences of mutations. Mutations may arise from a series of different mechanisms such as a direct chemical change in the DNA caused by reaction of the agent with the DNA, its precursors or the enzymes involved in DNA replication or repair, or through more genetically dependent mechanisms including gene conversions or movement of transposable elements. The relative roles that these mutational processes play in the production of spontaneous and induced mutations is not known. In addition, these events occur within a context of the physiological and metabolic processes of the mammal; a clear understanding and evaluation of somatic or germinal mutations requires that a proportion of our study be directed at in vivo mammalian mutagenesis.

Given the mutation frequencies and the number of useful markers, whole animal approaches would seem to have only marginal feasibility. However, data from microbial systems suggest the possibility that given an induced frequency and the number of mammalian genes, every offspring in an exposed population could be the carrier of a new mutation. Yet, our capacity to detect and understand these events in mammals is limited. Development of systems for detection of mutations in cells from the exposed individual (animal or human) would be extremely important for understanding the genetic damage caused by exposure to mutagenic agents and for elucidating mutation mechanisms in the mammal. Efforts are in two basic categories, 1) site specific alterations of proteins with detection in single cells and 2) site specific changes detectable directly at the DNA level.

OBJECTIVES OF RESEARCH PROJECT IN NEAR TERMS: There are plenty of methods for study of chromosome aberrations in single cells in vivo, but very few methods exist for obtaining point mutations or small intrachromosomal changes from single cells from laboratory mammals or humans. Study of the relationship of mutations to the organization of the genome may require isolation of many different types of mutations within at least a few genes. Using single cells obtained from the exposed individual may be the best approach to obtain a broad spectrum of mutations. Different genetic markers are being explored and evaluated for their suitability in these studies.

EXPERIMENTAL APPROACHES AND SCIENTIFIC JUSTIFICATION: The following approaches are taken. a) Use of altered proteins as markers for genetic changes in single cells. Selection of new markers for study of mutagenesis in single cells in the exposed individual is an important step. The past experience in this area has provided valuable information for guiding the selection. At present, the MOD-1 locus seems a promising candidate in mice. Use of this marker has the following advantages. (a) Transmissible mutations induced by ENU have been isolated. (b) The enzyme is present both in germinal and somatic cells. (c) Electrophoretic differences exist between various mouse strains, and (d) the mutants isolated after ENU treatment are likely to be due to single base-pair changes. An important feature is that heterozygotic animals carrying a wild-type allele and a

mutant allele can be produced. Cells from the heterozygote should simulate the mutant cells in the exposed animal. The antibodies, therefore, can be adjusted to detecting these cells, and the entire approach thoroughly tested. This was one of the essential lacking features of the LDH-C system. If antibodies can be isolated which are specific to the mutants, it should be possible to detect mutation both in somatic cells and sperm. This would enable a comparison of somatic, germinal, and transmissible mutation rates. Molecular analysis of the transmissible mutants will be attempted. The search will continue for other markers. Any individual marker which is selected may possess some unforeseen technical difficulties. Therefore, it is important that several markers are simultaneously developed. The other marker for which initial development will be started is glucose phosphate isomerase (GPI). This marker has some of the same salient features as MOD-1. b) Use of viral transformed cells for study of site specific mutations in mammals. Mouse L-cells were transformed with double stranded RF forms of ϕx 174 am3 cs70. Both markers can be used for study of reverse mutations. The phage DNA can be isolated from the mammalian DNA and be used for transfection of spheroplasts. It should be possible to study mutation induction in the viral DNA which is incorporated into the mammalian DNA. This DNA is exposed to the same metabolic activation enzymes and repair enzymes as the normal mammalian DNA. Although the viral DNA lacks some features of mammalian DNA such as repetitive DNA, it nevertheless should be possible to obtain accurate mutation information at specific sites. c) Use of mitochondrial DNA for study of genetic damage. The mitochondral DNA (mt-DNA) in mammalian organisms has features which are different from the nuclear DNA. The mt-DNA is circular, evolves faster, is less repaired, and usually shows maternal inheritance. Genetic diseases are known in humans which are traceable to mutations in the mitochondrial DNA. Analysis of mitochondrial DNA from sperm of mutagen treated and untreated male mice is one of the approaches taken and is related to the following observation: α-glycerophosphate dehydrogenase (αGPD) is a nuclear coded enzyme located between the inner and outer membrane of mitochondria. A high frequency of sperm from mice treated with certain mutagens lack α-GPD activity. The frequence varies with the treated stage of spermatogenesis. The highest frequency coinsides with the replication of DNA in the spermatids. One possibility is the lack of αGPD activity may be related to damage to the mitochondrial DNA. The DNA from sperm treated in different stages of the spermatogenesis will be analysed by restriction digests.

RECENT ACCOMPLISHMENTS AND SIGNIFICANCE: a) The mod-1 and gpi markers for single cell in vivo mutagenesis: The normal mod-1 enzyme has been purified and antibodies produced. These antibodies have been used to show that sperm as well as somatic cells will react to the antibodies. This is an important first criteria for the possibility of use as a genetic marker for detection of induced mutations. One form of abnormal MOD-1 has been test purified on a small scale from mice bearing the mutation in homozygous form.
b) The viral transformed L-cells: Preparation of spheroplasts are laborious and time consuming and the competence of the spheroplasts varies considerably between preparations. In order to circumvent this problem, a technique was developed to maintain their competency for at least nine months. DNA from mouse L-cells transformed with ϕX174 was digested with the restriction endonuclease pst-1 and treated with T_4 ligase. This DNA was used for transfection of

spheroplasts and ϕX174 plaques were recovered from this DNA. We will develop techniques for isolating the ϕX174 DNA from mammalian cells and in the future treat the L-cells transformed with ϕ174 am_3 cs_{70} with various mutagens. Reverse mutations of the am_3 and the cs_{70} will be measured among the phages recovered from DNA from treated and untreated cell cultures. c) Mitochondrial DNA: Mitochondrial DNA has been isolated from sperm taken from the vas deferens of a single mouse. Restriction patterns in this DNA have been visualized in mt-DNA from one hundreth of the DNA from sperm in one sample from one mouse. Cloned mt-DNA has been accumulated. The sensitivity of the system may enable us to detect changes in the mt-DNA after mutagenic treatment. If this system is successful it should open up the possibility for study of damage of the mt-DNA in mammals in vivo.

PLANS FOR SUBSEQUENT YEARS: a) The mod-1 and gpi markers for single cell in vivo mutagenesis: The genetic damage in the mutants will be studied of the molecular level, the results will be compared to both spontaneous mutations and induced mutations in other markers. This will indicate if there is any unique features on the mammalian genome affecting mutation spectrum. Attempts will be made to obtain heterologous antibodies to both wildtype and mutant proteins. In collaboration with Dr. Lohman (Netherlands) we hope to obtain monoclonial antibodies. Attempts will be made to adopt the system for the cell sorter. The enzymes are present in both sperm and somatic cells. If the approach is successful then data can be obtained for mutation frequencies in various tissues; it will also be possible to compare transmissible mutation rates, and single cell mutation rates in somatic and germinal tissue. These data may assist evaluation of mutation monitoring data from the human population.
b) The viral transformed L-cells. Attempts will be made to concentrate the viral DNA (ϕX174) from the mouse L-cells. If this is successful, ϕx174 will be recovered from mutagenic treated and untreated cells. In collaboration with the scientists at UNC, attempts are being made to transfect fertilized mouse eggs with ϕX174 which hopefully will result in mice with ϕX incorporated into their genome. Attempts will also be made to utilize other viral vectors such as lambda phage. This project may result in a system where site specific mutagenesis can be measured in any organ of a mouse from which DNA can be obtained in sufficient quantities. If successful, it may also enable use to understand mutation fixation in mammalian genome. It may also give very precise data of the sensitivity of the various sperm stages to mutagenic treatment. Knowledge of parameters about mutagenesis in a mammal is likely to give information about the damage induced by exposing the mammalian organism to a mutagen.
c) The mt-DNA: If the hypothesis that the lack of α-GPD activity in mutagen treated animals indicates damage to the mt-DNA, then it should be possible to evaluate the potential effect of mutagens on mt-DNA. The DNA will be cloned and restriction mapping (tentatively) used to identify the damage at the molecular level. Attempts will also be made to compare the profile of the proteins from treated and untreated animals. The sperm from the F_1 generation originated from either treated males or female will be studied for damage in the mt-DNA.

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LABORATORY OF MOLECULAR BIOPHYSICS

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SUMMARY STATEMENT

The goals of the Laboratory of Molecular Biophysics are to develop, improve and utilize spectroscopic methods to monitor the molecular interactions that occur between environmental agents and biological systems, to develop, improve and utilize analytical methodology for specified chemical agents, and to conduct biochemical, physical organic and bio-organic studies of environmental agents and their conversion products with emphasis on biomechanism elucidation. The Laboratory is organized into five separate Workgroups: Molecular Biophysics, Mass Spectrometry, Bio-organic Chemistry, Nuclear Magnetic Resonance, and Prostaglandin Biochemistry.

MOLECULAR BIOPHYSICS

The Molecular Biophysics Program is concerned with understanding, at the molecular level, the interaction of environmental agents with target biological systems, including nucleic acids, proteins and membranes. For these studies a number of highly sophisticated spectroscopic techniques (electron spin resonance, fluorescence and absorption spectroscopy, circular dichroism and stopped flow spectrometry) are employed. Particular emphasis is placed on the detection and identification of reactive free radicals (including active oxygen species) generated from environmental chemical agents by metabolic and other processes. There is now convincing evidence that free radicals are involved in a number of pathologic conditions including chemically induced carcinogenesis, pulmonary fibrosis, methemoglobinemia, hemolytic anemia and photosensitization.

Before free radicals can be clearly implicated in any toxic effect, the free radical species must be demonstrated to exist under appropriate biological conditions. Studies have continued to detect, identify and quantitate free radicals generated during the metabolism of xenobiotics. Benzidine has been shown to undergo oxidation by horseradish peroxidase and prostaglandin synthase to a radical cation and a charge-transfer complex formed between benzidine and its two-electron di-imine oxidation product. The di-imine is a resonance structure of the nitrenium ion, the proposed ultimate carcinogenic metabolite of aromatic amines. Horseradish peroxidase also oxidizes acetaminophen to a transient phenoxy radical, N-acetyl-p-benzoquinoneimine, which may arylate tissue macromolecules and cause hepatic necrosis. Sulfur-centered (thiyl) radicals have been detected when either cysteine or reduced glutathione was incubated with horseradish peroxidase. Since these compounds play an important role in the structure and function of sulfhydryl-containing proteins, these oxidation reactions may modulate the biological role of cysteine and reduced glutathione.

Studies on the formation of free radical intermediates from antiparasitic drugs have continued. Trypanosoma cruzi (the agent of Chagas' disease) has been found to reduce crystal violet to a carbon-centered radical which has been implicated in the biological activity of this triarylmethane dye. The free radical intermediates generated by polymorphonuclear leukocytes in the presence of antibody coated T. cruzi were identified as superoxide and the hydroxyl radical. Evidence has also been obtained for the one-electron reduction of SO_2, O_2, nifurtimox, nitrofurantoin and metronidazole by a variety of biological systems.

Light is known to interact with chemical agents in tissues, such as the skin or eyes, to produce photosensitization. The chemical agent may be endogenous (protoporphyrin in erythropoietic protoporphyria), a drug (sulfonamides, declomycin, chlorpromazine), a topical agent (4-aminobenzoic acid and its esters in sunscreens; halogenated salicylanilides in soaps) or an environmental agent (polycyclic aromatic hydrocarbons in coal tar; amyl esters of 2-aminobenzoic acid in printer's ink). The photosensitivity response may be one of two types, phototoxic or photoallergic. While the initial step in all forms of photosensitivity must be the absorption of light by the chemical or its metabolites, the precise mechanisms of photoxicity and photoallergy are unknown. Evidence has been sought for the involvement of free radicals and active oxygen species in photosensitization. The irradiation of benoxaprofen, an antiinflammatory agent which causes acute phototoxicity, in anerobic organic solvents resulted in hydrogen abstraction reactions and the formation of a drug-induced carbon-centered radical. In the presence of oxygen both superoxide and singlet oxygen were generated. Oxygen markedly increased the photohemolysis of erythrocytes by benoxaprofen suggesting that oxygen-derived species were involved. Anthracene and fluoranthene, the two major polycyclic aromatic hydrocarbons present in coal tar, are both potent phototoxic agents both in vivo and in vitro. In mice anthracene is photocarcinogenic, while fluoranthene is known to be a cocarcinogen. Irradiation studies in organic solvents and an erythrocyte model system identified singlet oxygen as the causative agent in the phototoxicity of these compounds. Light induced free radicals or oxygen-derived species were also detected with benzoxazole, musk ambrette, chlorpromazine, furosemide and several salicylanilide derivatives.

MASS SPECTROMETRY

The mass spectrometry program continues to develop new and improved analytical methods to facilitate all aspects of environmental/biological research. Advances in biomedical sciences have usually followed the development of analytical methods. State of the art instrumentation, such as the high resolution and tandem quadrapole mass spectrometers, is used to achieve high sensitivity and specificity in quantitative analysis. A new ion source, using negative primary ions for liquid secondary ion mass spectrometry, has been designed and constructed. By using a double focusing mass spectrometer, equipped with a highly focused point source and moveable target, it has been possible to determine the surface distribution of organic chemicals with a spatial resolution of less than one micron. The first tandem high resolution mass spectrometer, built to specifications of scientists in the Mass Spectrometry Workgroup, has been installed. This instrument permits the simultaneous high mass resolution of both parent and daughter ions at good sensitivity. Initial studies have demonstrated the power of this spectrometer for the characterization of peptides, corticosterone metabolites and phospholipids. The use of kinetic energy release measurements has been extended to the characterization of unknown organic compounds. Finally a new time-of-flight mass spectrometer has been designed which offers resolution equal to or better than that achieved when magnetic instruments are operated at very high mass (i.e. >8000 daltons).

Studies in gaseous ion chemistry have continued. Specific accomplishments include: demonstration of two structures in the cluster ion of $(NaF)2Na^+$;

measurement of the conversion of kinetic energy into internal energy for the molecular ion of 2-pentanone; demonstration of a Hammett correlation for ratios of intensities of collision-induced fragments on the basis of the statistical theory of mass spectra and in practice. The reaction paths leading to the formation and subsequent decomposition of PO^{3-} were determined from negative chemical ionization studies of a group of dimethylvinyl and dimethylaryl phosphates. Finally, the mechanism for the substitution reactions of NH_3 in the ammonia chemical ionization mass spectra of aromatic esters has been elucidated.

The Mass Spectrometry group has also analyzed more than 2000 samples in support of other NIEHS scientists. In addition a number of collaborative projects were carried out including the identification and determination of metabolites of phthalates and diethylstilbestrol; measurement and determination of chlorinated dibenzo-p-dioxins and dibenzofurans in soil and animal tissues; the distribution of 3-methyl-4-hydroxyphenylglycol in rat brain; the identification of physalemine-like peptides and epidermal growth factor in tissues; measurement and determination of corticosterones in the mammary gland; and the characterization of phospholipids.

BIO-ORGANIC CHEMISTRY

The Laboratory's bio-organic chemistry program is concerned with the development of methodologies to detect and identify metabolites of environmental chemicals in biological systems. Research is also carried out on the mode of interaction of environmental agents with biological systems at the molecular level with particular emphasis on metabolic factors.

The undesirable biological effects of phthalate esters (the most ubiquitous of all environmental pollutants), which include acute testicular atrophy resulting in male sterility, hepatocarcinogenesis in rats and mice, and proliferation of peroxisomes, are mediated through their metabolites. Some twenty seven metabolites of the most widespread phthalate [di-(2-ethylhexyl)phthalate] have been identified in several mammalian species and a metabolic pathway for their formation has been postulated. As the biological activities of the different metabolites are elucidated, it becomes clear that species differences in metabolism can potentially result in the resistance of non-rodent species to the undesirable biological effects. This renders extrapolation of toxicity tests from rodents to man high unreliable.

A major effort this year has been concerned with development, validation, and application of methods for the reliable determination of chlorinated dibenzo-p-dioxins and dibenzofurans at low levels in environmental samples. Emphasis has been placed on accurate quantification and highly specific qualitative identification. Methods for extraction and cleanup of soil, adipose tissue, and liver have been developed and refined. A collaborative study has been started, involving ten laboratories in three countries, to compare the analytical capabilities of these laboratories (which all use different techniques and approaches) for the low part-per-trillion level measurement of a series of dioxins and furans in human adipose tissue. The most reliable analytical approaches should be identifiable as a result of this study. Animal tissues and soil were analyzed for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in another study designed

to evaluate the bioavailability of TCDD bound to soil from Times Beach, Missouri. A new project in which the tumor promoter or antipromoter effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and polychlorinated biphenyls are compared to that of the phorbol esters is in the planning stage.

Other research has focused on the description of mechanisms at various biochemical and molecular levels including development of structure-activity correlations as a predictive tool in toxicology. Halogenated aromatic hydrocarbons constitute a broad class of compounds with varying structure and toxicity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the prototypical structure, is the most toxic compound of this type. Theoretical, quantitative structure activity relationship (QSAR) and experimental studies focusing on polychlorinated biphenyls (PCBs) as models have established the importance of molecular polarizability and receptor to PCB separation distance in binding to the dioxin or Ah receptor. The work suggests a new stacking type model for the Ah receptor which has universal applicability to the range of binding-structures observed. Further theoretical and experimental studies suggested that Ah receptor binding is a necessary but not sufficient condition for toxicity and a second receptor must be proposed to account for the structural requirements of planarity and halogenation in toxicity, as opposed to induction. A protein binding model consistent with the structural requirements for toxicity has been identified. Molecular modeling work, competitive binding assays and several in vivo studies have demonstrated the importance of thyroxine binding proteins in toxicity and suggested that the high toxicity of TCDD is the expression of potent and persistent thyroid hormone activity. A molecular mechanism has been proposed involving a two protein-receptor model in which the planar aromatic system controls the initial receptor binding and halogen substitutents control subsequent nuclear events. This mechanistic hypothesis is being further investigated for its relevance in dioxin toxicity, as well as insights into the mechanism of thyroid hormone action.

The Laboratory's organic chemistry program develops new and improved methods in synthetic chemistry to support diverse studies on biomechanism elucidation at the molecular level with particular attention to stereochemistry. Emphasis is placed on the identification of reactive electrophilic species from metabolic activation that may mediate toxic effects. Of particular interest has been the metabolism of polycyclic aromatic hydrocarbons and detoxification by the glutathione S-transferase enzymes. The idea that the metabolism of chemicals in the body can lead to the formation of more toxic substances, eg. epoxides, through metabolic activation or less toxic substances through detoxification has been widely accepted. The mechanistic aspects of the GSH transferase reaction underscore the relevance of stereochemical factors in the influencing the rate of elimination of enantiomeric oxides via the glutathione pathway. Specific accomplishments include the development of methods which allow the preparation of diastereomerically pure glutathione conjugates as well as sensitive methods for the analysis of such compounds. The stereochemical course of the epoxide ring opening step was established with the aid of optically pure epoxides. This knowledge has been translated into stereochemical profiles whereby diastereomer identification on a reversed-phase liquid chromatography system became possible. Experiments with various epoxide substrates and hepatic glutathione transferases from the little skate and the rat showed remarkable stereochemical consistency.

In both cases, a majority of the purified enzymes showed a high preference for attack-addition of thiol at the R-carbon on the epoxide ring. Other enzymes showed the opposite preference, i.e. addition to the S-carbon. A mechanism has been postulated to explain the stereoselectivity of this enzymatic transformation and, based on this mechanism, a model has been proposed for the active site geometry of three glutathione transferases. The use of stereochemical profiles as a way of assesing differences, or similarities, among glutathione transferases is currently being explored.

Work has continued on the development of analytical method for the analysis of biologically active compounds. The high-performance liquid chromatography analysis of peptides has focused on the role of hydrophobicity in the separation mechanism by reversed phase liquid chromatography. Experiments have been designed and implemented which alter the intrinsic hydrophobicity of peptide molecules during chromatographic analysis. These changes are introduced in a predictable fashion such that valuable structural information is derived from conventional reversed-phase experiments. Successful applications of this approach include: characterization of amphibian peptides in small-cell carcinoma, purification of a protein seemingly synthesized in response to estrogen exposure, and characterization of two forms of epidermal growth factor isolated from mouse submaxillary gland.

NUCLEAR MAGNETIC RESONANCE

The objective of the biophysical nuclear magnetic resonance (NMR) program is the elucidation of the mechanisms by which chemicals and heavy metals present in the environment produce toxic effects in living systems. The use of NMR methodology in achieving this objective may be considered to fall into two broad categories: 1) In vivo metabolic analysis using NMR spectroscopy. Such studies probe the metabolism of chemicals and heavy metals in various tissues and organs directly when sufficient concentrations are present to permit detection. Additionally, studies of the effects of these agents on intermediary metabolism, the metabolism of high energy phosphate compounds, and on the pyridine nucleotide reduction charges are carried out when the levels of chemicals/heavy metals are well below the direct detection threshold, but can still lead to a toxic response as reflected in perturbations of those parameters. 2) In vitro studies of molecular structure and dynamics using NMR spectroscopy. Such studies are aimed both at obtaining a more complete understanding of the solution structure of chemical toxins, and toward the analysis of the mode of interaction of various toxic substances with presumed or demonstrated biological targets (e.g., nucleic acids, enzymes, etc.). As a consequence of the need to use specific labeling (including carbon-13 and fluorine-19 labeling) in connection with many of these NMR studies, the program includes a synthetic component, the goals of which are closely interfaced with the needs of the program. Finally, it is noted that an overriding motivation for this program is the evaluation and demonstration of the extent to which non-invasive analysis by NMR can be used to follow the progress of a toxic response in real time and with individual experimental animals, thereby leading to more efficient use of these animals in toxicity studies.

PROSTAGLANDIN BIOCHEMISTRY

The prostaglandin group investigates the metabolism of arachidonic acid to prostaglandins (PG), hydroxy fatty acids (HFA) and leukotrienes (LT) and studies their role in a number of important physiological and pathophysiological events. The group also studies the metabolism of chemicals by prostaglandin synthase (co-oxidation) in order to determine the role of this metabolic pathway in the development of chemically induced carcinogensis.

The metabolism of aromatic amines by prostaglandin synthase (PHS) has been extensively studied. The bladder carcinogen 2-aminofluorene (2-AF) is metabolized to several unique metabolites by PHS which appear to be formed by free radical pathways; the corresponding hydroxylamine does not appear as an intermediate. PHS was also found to catalyze the formation of 2-AF-DNA adducts. Evidence was obtained for the formation of at least three different DNA adducts which were distinct from the adducts produced by activation of 2-AF by the mixed function oxidase system. We observed that the addition of phenylbutazone stimulated the epoxidation of benzo(a)pyrene-7,8 diol by PHS to the anti diol epoxide. This represents a new class of reactions catalyzed by PHS.

The metabolites formed from arachidonic acid by dog tracheal epithelial cells, mouse skin epidermal cells and rat alveolar macrophages have been characterized. Exposure of macrophages to asbestos resulted in the release of LTB_4 and LTC_4. The response appeared to be related to the binding of asbestos to cell membranes rather than ingestion of the particles. The role of arachidonic acid metabolites in controlling chloride ion secretion has been studied. Release of arachidonic acid and its metabolites was accompanied by an increase in Cl^- secretion. Indomethacin inhibited both Cl^- secretion and PG formation. PGD_2 was particularly effective in stimulating Cl^- secretion. LTC_4 also released PG from cells and thus indirectly altered Cl^- secretion. These studies suggest a role for arachidonic acid metabolism in controlling pulmonary secretion. The possible involvement of arachidonic acid metabolites in the etiology of cystic fibrosis is currently under study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 10003-05 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthetic and Analytical Studies in Bioorganic Chemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Oscar Hernandez	Senior Staff Fellow	LMB	NIEHS
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COOPERATING UNITS *(if any)*

Laboratory of Behavioral and Neurological Toxicology
Development and Reproductive Toxicology

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio Organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this project is to develop chemical methods and apply these to specific biological areas. Two problems of current interest are 1) chemistry of glutathione and its adducts with epoxides; 2) mechanisms of separation and purification of polypeptides by high-performance liquid chromatography. The level of success of a program on bio-mechanisms, such the one posed by the glutathione pathway, is largely dependent on the ability to generate the chemical information necessary for the interpretation of biological experiments. A systematic program was initiated and eventually has culminated in the development of unequivocal syntheses of the requisite compounds, methods of separation for diastereoisomeric conjugates of glutathione with alkene and arene oxides, establishment of the absolute configuration of these thioether stereoisomers, and correlation of synthetic compounds with enzymatically produced conjugates. The high-performance liquid chromatography analysis of peptides has focused on the role of hydrophobicity in the separation mechanism by reversal phase liquid chromatography. Experiments have been designed and implemented which alter the intrinsic hydrophobicity of peptide molecules during chromatographic analysis. These changes are introduced in a predictable fashion such that valuable structural information is derived from conventional reversed-phase experiments. Successful applications of this approach include: characterization of amphibian peptides in small-cell carcinoma, purification of a protein seemingly synthesized in response to estrogen exposure, and characterization of two forms of epidermal growth factor isolated from mouse submaxillary gland.

A. Synthetic and Analytical Studies in Bioorganic Chemistry

Nature of Problem: The level of success of a program in biomechanisms is highly dependent on the ability to generate the chemical information necessary for the interpretation of biological experiments. Because of this established symbiosis, the nature of this information is defined by the particular needs of the project at hand. In general, this program does not deal with the implementation of published procedures but rather with the development of synthetic and analytical methodology and with the application of these methods to specific biological problems. This project in its inception reflects the interactive nature of chemistry whereby ideas and knowledge originate in response to challenges for biological disciplines. Two areas of current interest are: 1) chemistry of glutathione and its adducts with epoxides; 2) mechanisms of separation and purification of peptides by reversed-phase high-performance liquid chromatography.

Objectives: The principal objectives in the glutathione project are 1) to design unequivocal synthetic routes leading to glutathione adducts of alkene and arene oxides; 2) to develop chromatographic procedures for the separation and purification of stereoisomeric glutathione conjugates of epoxides; 3) to establish the absolute configuration of diastereoisomeric glutathione conjugates produced in rats. The steps outlined above were implemented in order to ensure the chemical and stereochemical integrity of the synthetic samples. Based on this knowledge, meaningful correlations with enzymatic systems could be subsequently attempted.

For the peptide portion of the project, the focus was on the role of hydrophobicity in the separation mechanism of peptides by reversed-phase high-performance liquid chromatography. The hypothesis tested in this segment was that the disruption of the hydrophobic surface accessible for binding of a peptide molecule to a reversed-phase bonded column would result in increased or decreased retention in the chromatographic column, and that the direction of this change could be predicted based on the nature of the disruption introduced. The immediate benefit available from this approach would be increased selectivity in peptide separations; subsequently, the predictive value of these experiments could be extended to structural studies of peptides where the amounts of sample available severely limit the application of conventional techniques.

Experimental Approach: For the synthesis of glutathione adducts of epoxides two design features were particularly important. First, it was critical that the conditions used would not result in racemization of the resulting thioether conjugates. Second, that the anticipated mixtures should be produced in a form suitable for chromatographic purification. The first consideration required the initial development of a method to assess the extent of racemization of the peptide moiety, and based on these experiments a suitable base and solvent combination could be selected. The second consideration could be satisfied by using normal-phase chromatography, which would provide the capacity required for purification of relatively large sampler. This latter feature also dictated the use of a fully-protected, organic solvent soluble glutathione.

For the modification of selectivity during reversed-phase column chromatography of peptides, it became evident that pH was the logical variable to introduce

changes designed to alter the intrinsic hydrophobicity of peptide surfaces. For small peptides, up to fifteen amino acid residues, our initial efforts were concentrated on the presence of histidine residues as a way of altering hydrophobicity as a function of pH. At low pH the imidazole ring would be protonated thus disrupting the hydrophobic surface accesible for binding. At neutral pH this binding surface on the peptide molecule would be reestablished with a concomitant, and predictable, effect on column retention. For larger peptides, where conformational effects grow in importance, the effect of pH on selectivity was also deemed important based on the following argument. At neutral pH a number of amino acid residues participate in hydrogen bonding, salt bridges, etc. The combination of these interactions results in a preferred conformation(s). If, however, the pH of the solvent is lowered a substantial portion of these intramolecular interactions would be inhibited resulting in the adoption of a conformation different from that at neutral pH. The conformers obtained at acid and neutral pH would more likely differ in the nature and extent of hydrophobic surfaces accesible for binding and this in turn would result in increased selectivity during reversed-phase liquid chromatography. This concept has been validated by the separation and purification of two forms of mouse epidermal growth-factor.

Proposed Course: The proposed course of action involves completion of the glutathione project by including the conjugates derived from the 7,8,-diol 9,10-epoxide of benzo[a]pyrene and benz[a] authracenes 5,6-oxide.

Further development of the Mitsunobu reaction to provide access to thioether adducts of non-K-region arene oxides, a series of compounds of recognized importance but which so far remain inaccessible.

C. Publications of past 18 months:

Hernandez, O., Bhatia, A.V. and Walker, M.P.: HPLC separation of the diastereomeric glutathione adducts of styrene oxide. J. Liquid Chromatogr. 6: 1475-1489, 1983.

Hernandez, O. and Kohli, K.K.: Removal of the nonionic detergent Emulgen 911 from solubilized microsomes by HPLC. J. Liquid Chromatogr. 6: 1459-1473, 1983.

Hernandez, O., Bhatia, A.V. and Walker, M.P.: HPLC separation of diastereomeric adducts of glutathione with some K-region arene oxides. J. Liquid Chromatogr. 6: 1693-1704, 1983.

Hernandez, O., Dermott, K., and Lazarus, L.: High-performance liquid chromatography of amphibian peptides. Selectivity changes induced by pH. J. Liquid Chromatogr. 7: 893-905, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES10004-05LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies in Nuclear Magnetic Resonance (NMR) Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert E. London	Research Physicist	LMB	NIEHS
OTHER:	Anthony Ribeiro	Chemist	LMB	NIEHS
	Louis Levy	Research Chemist	LMB	NIEHS
	Halasya Ramanathan	Visiting Fellow	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Nuclear Magnetic Resonance Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	3.2	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of the biophysical nuclear magnetic resonance (NMR) program is the elucidation of the mechanisms by which chemicals and heavy metals present in the environment produce toxic effects in living systems. The use of NMR methodology in achieving this objective may be considered to fall into two broad categories: 1) In vivo metabolic analysis using NMR spectroscopy. Such studies probe the metabolism of chemicals and heavy metals in various tissues and organs directly when sufficient concentrations are present to permit detection. Additionally, studies of the effects of these agents on intermediary metabolism, the metabolism of high energy phosphate compounds, and on the pyridine nucleotide reduction charges are carried out when the levels of chemicals/heavy metals are well below the direct detection threshold, but can still lead to a toxic response as reflected in perturbations of those paramters. 2) In vitro studies of molecular structure and dynamics using NMR spectroscopy. Such studies are aimed both at obtaining a more complete understanding of the solution structure of chemical toxins, and toward the analysis of the mode of interaction of various toxic substances with presumed or demonstrated biological targets (e.g., nucleic acids, enzymes, etc.). As a consequence of the need to use specific labeling (including carbon-13 and fluorine-19 labeling) in connection with many of these NMR studies, the program includes a synthetic component, the goals of which are closely interfaced with the needs of the program. Finally, it is noted that an overriding motivation for this program is the evaluation and demonstration of the extent to which non-invasive analysis by NMR can be used to follow the progress of a toxic response in real time and with individual experimental animals, thereby leading to more efficient use of these animals in toxicity studies.

A. Studies in Nuclear Magnetic Resonance (NMR) Spectroscopy

Nature of Problem: One of the principal limitations on attempts to elucidate the mechanisms of chemical toxicity at the molecular level is the need to deal with isolated cellular components. Indeed, the reductionist strategies of traditional biochemical analysis which have proven so fruitful are all eventually limited to extrapolation from an in vitro model. This extrapolation may be most misleading if the toxic response involves primarily a perturbation of the regulation of cellular metabolism, since it is particularly difficult to obtain meaningful regulatory data with in vitro or even in situ systems such as perfused organs. During the past decade, an alternative NMR approach has been developed which permits the analysis of cellular metabolism in vivo and can be extended to intact experimental animals. There are some significant limitations of this strategy which exist at present - particularly the limits of sensitivity which restrict studies to relatively concentrated metabolites. Nevertheless,the ability to get away from in vitro models and deal with intact systems opens a new dimension in biochemical analysis. Additionally the development of non-invasive probes for the metabolic response of an experimental animal to a chemical toxin could become a significant tool of toxicological analysis, leading in particular to a more efficient use of experimental animals.

As a consequence of the sensitivity limitations of the NMR technique noted above, not all questions are amenable to this technique. We can, however, address several specific problems:

1. Acute toxicity reactions can reflect disruption of intermediary cellular metabolism. For example, proper cellular function requires that ATP levels, and redox levels of pyridine nucleotides be maintained within narrow limits. Perturbations of these concentrations, or of carbohydrate or lipid metabolism required to maintain these metabolite concentrations, can lead to a rapid toxic response. The capability of the NMR method to evaluate a variety of parameters related to intermediary metabolism is well established, and this represents one general approach to the analysis of acute chemical or heavy metal toxicity in vivo. As a specific example, we note that Cohen et al. (Proc. Nat. Acad. Sci. U.S.A. 78: 60, 1981) have observed changes in the rate of gluconeogenesis from carbon-13 labeled alanine in rats made hyperthyroid by treatment with triiodothyronine. This study has particular relevance to the development of an understanding of the toxicological response to tetrachlorodibenzo-p-dioxin since there is evidence to suggest that this chemical may be thyromimetic (J. McKinney, private communication).

2. A second area of application of the NMR technology concerns the determination of the physical state and/or metabolic fate of particular chemicals. The NMR signals corresponding to particular intracellular chemicals or metabolites are modulated by the physical state of these chemicals. Association with macromolecules can lead to perturbations of relaxation time or chemical shift which allow an analysis to be made in the intact organism. Some metabolic transformations of isotopically labeled chemicals or fluorinated chemicals can also be monitored. Although frequently the concentration of such metabolites is low, we propose to utilize the traditional toxicological strategy of simulating the effects of a prolonged, chronic low exposure by the use of a single, large dose. This approach represents a common strategy of both NMR and toxicological

research. A particular class of compounds of interest from this standpoint are the herbicides many of which are fluorinated. Particular herbicides of interest from the standpoint of toxicological analysis by NMR include trifluralin and others which contain a CF_3 substituent on an aromatic ring, hexafluoroarsenate, as well as phosphorus containing herbicides such a glyphosphate and ethepon (Herbicide Handbook of the Weed Science Society of America). All of these herbicides are considered to be essentially non-toxic, and rats have been fed extremely high doses (typically ~ grams/kg) without a measurable toxicological response.

3. The above discussion alludes to the use of NMR as a non-invasive probe of metabolic function. The development of the methodology for analysis must be geared to instrumentation development, particularly from the standpoint of increasing the selectivity of the NMR observations and dealing with such undesirable problems as radiofrequency heating of the sample (animal) which becomes a particularly significant problem in carbon-13 NMR studies which require proton decoupling. This requires parallel methodological development with the objectives of: increasing the selectivity of the observation; reducing undesirable rf heating effects; carrying out simultaneous observations of more than one nuclear species; development of additional strategies for monitoring metabolic function.

As a consequence of the need to use specific labeling (including carbon-13 and fluorine-19) in connection with many of the NMR studies, the program includes a synthetic component, the goals of which are closely interfaced with the needs of the program. Initial synthetic effort is being focused on carbon-13 labeled nicotinic acid, a precursor used to label the pyridine nucleotide pools, on the synthesis of a carbon-13 labeled ascorbate which will be used for in vivo studies of vitamin C metabolism, on [3-^{13}C] cysteine which is of interest in connection with glutathione and taurine metabolism, and on specifically labeled aspartic acid for metabolic studies of the liver and heart. Initial synthetic efforts with fluorine-19 will be focused on a fluorinated chelate (Smith et al., PNAS 80: 7178, 1983) used to study intracellular calcium levels.

Objectives: The objectives of this activity include the development of methodology for monitoring metabolic function in the various organs and tissues of the intact experimental animal with or without exposure to environmental significant chemicals. This goal requires that methodological development and particular application go hand-in-hand. Primary focus of the metabolic studies is on the liver as a consequence of its central role in detoxification, and on the heart using a perfused organ approach under development by the group. Ultimately, it is hoped that this approach will enhance our understanding of various mechanisms involved in chemical toxicity at the molecular level, particularly those which alter metabolic regulation. Since NMR is rapidly evolving into a major clinical diagnostic tool, it is anticipated that the identification of NMR detectable responses to chemical toxins may ultimately have clinical utility for the diagnosis of exposure of humans to chemical toxins. Finally, the development of non-invasive strategies for the analysis of toxicity in experimental animals could lead to the more efficient use of these animals in toxicological studies.

Experimental Approach: The primary approach for tissue selective spectroscopic analysis at present involves the use of "surface coils" which can monitor tissues directly beneath the coil. Since, as the name implies, the sensitivity is greatest closest to the surface, the method is most suitable for studies of muscle metabolism, as has been elegantly demonstrated in the laboratory of George Radda. In order to study hepatic metabolism, our group has recently developed a surgical approach to remove the layer of abdominal muscle of the rat located directly above the liver. This permits the observation of liver metabolic changes in the intact animal.

Although the NMR method can in principle and in practice be used to study the metabolism of a perfused heart placed in an NMR tube, we believe that such measurements are of very limited value in the absence of simultaneous monitoring of heart function. We have recently developed an approach to monitor simultaneously both, as described in the following section.

The goal of obtaining spectral data from a well defined, spatially localized volume of tissue represents an important determinant of the research effort in this field. The surface coil itself can be used to obtain some one dimensional "imaging" type information based on the inhomogeneity of the B_1 field which it produces. As discussed below, we will be modifying the basic design in order to enhance this spatial selectivity. Alternatively, Aue et al. (J. Magn. Reson. 56: 350, 1984) have recently described what appears to be the most general and powerful method of obtaining localized spectral data. In this method, switched field gradients as used for imaging are employed to excite a selected volume of tissue. Hence, in contrast to the surface coil approach, the selection is in the excitation rather than in the detection. We hope to work with this technique, contingent on the purchase of an instrument with switched field gradient capability.

Recent Accomplishments and Future Plans: Several preliminary studies of hepatic metabolism in the intact rat have been reported to date. All of these have been limited by the need to carry out a selective observation on the liver tissue. This is most seriously hampered by the surrounding layers of muscle tissue which also contribute resonances to the observed spectrum. The muscle contribution is readily detected by the phosphocreatine signal which is absent in the liver. In several of the studies described, the organ has been exposed prior to the measurement. The surgical trauma associated with this approach is extreme, and the animal cannot be studied over a period of time. We have recently developed an approach involving the surgical removal of a portion of the abdominal muscle directly above the organ. In this procedure, the skin is sutured closed after removal of the muscle, so that the rat has in effect a surgically created hernia above the liver. The liver of the rat thus modified can be observed over an extended period of time. Initial studies to evaluate the approach have been carried out on the effect of a large infusion of fructose (250 mg/kg) which produces dramatic changes in the high energy phosphate metabolites as reflected by NMR. These include the dramatic increase in the concentration of fructose-1-phosphate, an initial decrease followed by a large increase in inorganic phosphate, and a decrease in the level of ATP which recovers very slowly. Initial studies of the effects of a large dose of carbon

tetrachloride (150 ml/kg) have also been carried out, and a loss in intensity of the high energy phosphate resonances observed over a period of hours. Additional studies of this effect are in progress.

NMR studies of cardiac metabolism: Although the heart from a small experimental animal can be excised and perfused for study in an NMR tube, such measurements are of limited interpretive use of in the absence of simultaneous measurements o cardiac function. One approach to this problem has been the insertion of a balloon into the left ventricle. This approach has several limitations which include the difficulty of the surgical procedure on a heart which must be sufficiently small to fit the NMR coil, and the production of interfering resonances from the balloon. We have recently carried out a project to combine the NMR measurement with an approach which monitors heart contractions on a physiological recorder. In this approach, the perfused heart is hung at the end of a column of perfusate which is water jacketed for temperature control. A clip is applied to the heart and the duration and intensity of contractions monitored on a recorder. We have designed an NMR probe around this model which can simultaneously monitor phosphorus metabolite levels in the perfused heart. Development work on this project is nearly completed, and we hope to begin measurements in the near future. Subsequently, we will evaluate the effects of a variety of chemical toxins and proposed treatments on cardiac function and metabolism. One particular example of interest concerns the effects of a pharmacological dose of ribose which has been proposed to play a cardioprotective role. This use is based on the fact that the ribose metabolite 5-phosophoribosyl-1pyrophosphate (PRPP) is an essential substrate for the synthesis of purine and pyrimidine nucleotides. Thus, it has been experimentally verified that ribose enhances cardiac adenine nucleotide biosynthesis in animals recovering from oxygen deficiency and experimental myocardial infarction. The effects of ribose administration are, however, expected to differ in the liver relative to the heart as a consequence of the greater availability of PRPP in the former organ due to the greater activity of the oxidative pentose phosphate pathway which ultimately converts ingested glucose into PRPP. It is clearly of interest to determine, using the in vivo methodology described here, to what extent the administration of ribose will stimulate the biosynthesis of ATP both heart and liver and in how all of the high energy phosphate pools will be altered by such administration. Beyond the evaluation of the effects of how this proposed cardioprotective application might produce additional changes in liver metabolism, it appears that the ability to increase hepatic ATP levels could potentially have some relevance in the protection of liver against a wide variety of toxic agents which reduce these levels.

In vitro NMR: In addition to the new initiatives in in vivo and in situ metabolic analysis, more traditional solution state NMR studies have been carried out on a variety of compounds related to environmental health. During the past year, a series of structural studies of reduced benzanthracenes, aza aromatics, and glutathione adducts of benzo[a]pyrene have been carried out. Studies of the ring conformations of the reduced benzanthracenes have been carried out, and data has been gathered on the relationship between chemical shift and the position of the nitrogen in 1-aza and 4-aza phenanthrene and in 1-aza benzanthracene. Two

dimensional NMR studies have proven of particular value for making the resonance assignments in these systems. Preliminary studies have also been made of cadmium binding phosphogycoprotein, cadmium-mytophosphatin. This unique protein derived from the wild mushroom Agaricus, differs from the more studied metallothioneins since it lacks cysteine residues. Instead, the protein is phosphorylated and contains large amounts of the acidic amino acids glutamate and aspartate. Presumably, these are involved in complexation with the cadmium.

Construction of a ^{19}F Surface coil; Observation of Free Cellular Cadmium Calcium: During the past year, we have constructed a surface coil for the ^{19}F nucleus, and will be iterating the design in the next fiscal year. We plan to begin observations of the ^{19}F resonances of several herbicides administered by injection to experimental rats in order to study hepatic metabolism and physical status. It should be particularly interesting to observe the apparently non-toxic hexafluoroargenate resonances in the rat liver. A second application of fluorine-^{19} NMR concerns the determination of the concentration of intracellular calcium levels. Smith et al. (PNAS 80: 7178, 1983) have recently described the use of fluorinated chelators which can be used to determine levels of calcium ions in vivo. As a consequence of the important regulatory role which free calcium ion concentration is believed to play on a broad spectrum of cellular processes, such information would be of particular value in studies of the toxicity of chemicals, and particularly heavy metals which may in some instances compete with various calcium binding sites of cellular proteins. These studies will be carried out with Dr. Elizabeth Murphy, who will join the group in October as a Senior Staff Fellow.

Carbon-13 and Phosphorus-31 NMR Studies of fatty degeneration of the liver: Fatty degeneration of the liver in response to toxins. Fatty degeneration of the liver is a common response to a variety of toxins including carbon tetrachloride, ethionine, ethanol as well as a broad range of chlorinated aromatic compounds including 2,3,7,8-tetrachlorodibenzo-p-dioxin. A closer analysis indicates, however, that the nature of the alterations in lipid metabolism and distribution accompanying the exposure to these toxins differs substantially from one case to the next. Thus, in some cases the triglycride pools are selectively increased, in other cases redistributed, and in a few cases, phospholipids also accumulate. It has been demonstrated that, as a consequence of the relatively high level of lipid molecules in most cells and their relative mobility, carbon-13 NMR is able to detect these lipid resonances at natural abundance. Among the parameters most readily determined are indices of lipid "mobility" as reflected in the linewidths and relaxation parameters of the resonances, degree of unsaturation - which corresponds to uniquely shifted resonances, as well as information about the nature of polar head groups in phospholipids or the concentration of triglycerides based on the analysis of the resonances of the glycerol moiety. Additional information on the distribution of intra- vs. extracellular compounds in general, and lipids in particular, may be obtained by the use of chelated shift reagents. These agents, which are basically lanthanides tightly chelated to tripolyphosphate or other negatively charged ligands, are apparently fairly non-toxic to a variety of cells, and also do not permeate the cell membrane. Consequently, they can be use to distinguish intra-from extracellular materials. In principle, such agents can be injected intraveously, and used to distinguish intra and extracellular lipids or other metabolites.

In addition to the characterization of the unlabeled lipids by ^{13}C NMR, studies of the various carbon-13 labeled toxins are also proposed. Many of these compounds, such as ^{13}C labeled carbon tetrachloride and [1-^{13}C] or [2-^{13}C] ethanol are currently available commercially. Further since one of the main effects of ethanol on the accumulations of triglycerides in the liver is presumed to be mediated by reduction of the NAD(H) pool, these studies can be carried out utilizing labeled nicotinic acid/nicotinamide to label the pyridine nucleotide pools so that the intracellular redox status can be monitored in parallel with the other biochemical transformations. Additionally, the unility of ^{13}C NMR for the study of gluconeogenesis in the perfused liver has been demonstrated by several groups. To date, no study has been carried out in the liver of an intact animal. It is proposed that, subsequent to our development of doubly tuned ^{13}C surface coil, analogous studies be carried out in the liver of the intact rat. Having demonstrated the feasibility of this approach to the study of gluconeogenesis in vivo, it will be possible to monitor the effects of various toxic chemicals and heavy metals on this process quantitatively.

In addition to the applications of carbon-13 NMR outlined above, phosphorus-31 NMR should prove useful in studies of the fatty degeneration of the liver outlined above. In addition to monitoring changes in the nucleotide triphosphate pools, the technique is sensitive to intracellular pH, and recently sensitivity to phospholipase activity, as reflected in the intensities of glycero-3-phosphocoholine and glycero-3-phosphoethanolamine resonances, has also been demonstrated.

Ascorbic Acid Metabolism: Ascorbic acid plays a principal role in the detoxification of environmental chemicals. Carbon-13 NMR has been used as a probe of ascorbic acid chemistry in vitro, and we propose to extend this approach to in vivo analysis. Based on the tissue concentrations anticipated which range from about 1 mM in the liver to 10 mM in brain and adrenal gland, these studies will require the use of carbon-13 labeled ascorbate. In particular, it is anticipated that the interconversions between ascorbate, ascorbate free radical, and dehydroascorbate can be monitored by this technique. Although the free radical would be expected to be present at much lower concentrations, chemical exchange between this species and ascorbic acid would significantly alter the relaxation parameters of the atter. Unfortunately the C-1 position which is the most readily labeled is the least useful for in vivo analysis as a consequence of its long relaxation time, so that it will be necessary to label one of the other carbons, preferably C-4.

Publication of past 18 months

Scott, L.T., Stang, T.H. and Levy, L.A. Automerizations in benzenoid hydrocarbons. New mechanistic insights from the thermal rearrangements of benz(a)anthracene-5-^{13}C. Tetrahedron Lett. 25, 1661 (1984).

Levy, L.A. "Synthesis of 2,3,6,7-tetrasubstituted naphthalenes: 2,3,6,7-tetrachloronaphthalene. Synthetic Communications 13, 639 (1983).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 10007-04 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

High Pressure Liquid Chromatography/Mass Spectrometry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Carol E. Parker	Research Chemist	LMB	NIEHS
OTHER:	J. Ronald Hass	Research Chemist	LMB	NIEHS

COOPERATING UNITS *(if any)*

Research Service Branch

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Mass Spectrometry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 50080-02 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 10012-02 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Design of Laboratory Data Management System

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Mike P. Moorman Biomedical Engineer LMB NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Mass Spectrometry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been discontinued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25012-05 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Lung as an Endocrine Organ Controlling Intravascular Thrombosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Thomas E. Eling	Research Chemist	LMB	NIEHS
OTHER:	Roberta McMillan	Biologist	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Prostaglandin Biochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 80008-10 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30003-13 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Analytical Methodology

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Phillip W. Albro	Research Chemist	LMB	NIEHS
Other:	J. Ronald Hass	Research Chemist	LMB	NIEHS
	Carol E. Parker	Chemist	LMB	NIEHS
	Kun Chae	Chemist	LMB	NIEHS

COOPERATING UNITS *(if any)*

EPA; Dow Chemical Company; Monsanto; New York Department of Health; University of Umea, Sweden; Health Prot. Br., Canada; National Fisheries Laboratory; Wright State University; University of Nebraska.

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	1.7	1.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major effort this year has been concerned with development, validation, and application of methods for the accurate and reliable determination of chlorinated dibenzo-p-dioxins and dibenzofurans at low levels in environmental samples. Emphasis has been placed on accurate quantification and highly specific qualitative identification. Methods for extraction and cleanup of soil, adipose tissue, and liver have been developed and refined. A collaborative, study has been started involving ten laboratories in three countries, to compare the analytical capabilities of these laboratories (which all use different techniques and approaches) for the low part-per-trillion level measurement of a series of dioxins and furans in human adipose tissue. The most reliable analytical approaches should be identifiable as a result of this study. Analysis of animal tissues and soil for 2,3,7,8-TCDD was used in another study to evaluate the bioavailability of TCDD bound to soil from Times Beach, Missouri.

A. Development of Analytical Methodology

Nature of Problem: Analytical data needed in support of biomedical research often exceeds present methodological capabilities. Development of adequate methodology is in itself a form of research, since the generation and interpretation of new types of data are essential to the process.

Objectives:

A. General: To develop and refine methodology for the qualitative and quantitative determination of compounds and classes of compounds of general interest to the Institute and special interest to individual investigators.

B. Current: (1) To develop reliable methodology for the extraction and cleanup of soil, adipose, and liver for reproducible and accurate qualitative and quantitative determination of part-per-trillion levels of chlorinated dibenzo-p-dioxins and dibenzofurans; (2) to organize and perform a round-robin, blind study to evaluate a wide range of procedures used in various laboratories for the determination of part-per-trillion levels of chlorinated dibenzo-p-dioxins and dibenzofurans in human adipose tissue.

Approach and Justification: Extraction and cleanup procedures, involving relatively rapid, multi-sample capabilities, are based on elutriation techniques and optimized chromatographic procedures. Recoveries and precision are evaluated through the use of isotopically labeled compounds. Emphasis is placed on preparation of validly spiked surrogates, in which it can be demonstrated that exogenous (spiked) analyte equilibrates with endogenous analyte. The participants in the round-robin study, the dioxin and furan standards, and the human adipose tissue were recruited or acquired through the EPA. Preparation of blind, spiked tissue extracts to be sent to the participants for analysis required objective evidence that the 10 sets of 10 samples each were spiked uniformly and correspondingly, and was handled by NIEHS. Information on amounts of adipose needed and limits of determination were supplied by the participants. Breaking the blind code and evaluation of the analytical results is the responsibility of NIEHS. Participating laboratories were selected because they represent the complete range of analytical approaches to this determination, have all acquired international reputations for their previous work on dioxin/furan analysis, yet have never participated in a comparison of capabilities in a blind study where the right answers were known by independent means.

Recent Accomplishments and Significance: (1) The extraction techniques that have been developed give at least 99% recovery of dioxins and furans from liver. Recoveries from adipose tissue range from 97.5 ± 3.6% (S.D.) at the ppb level to 85.1 ± 4.9% (S.D.) at the low parts per trillion level. The recoveries from soil depend upon the amount of organic mater (humus) in the type of soil analyzed, but clay content does not seem to influence the results. Recoveries from 93-99% can be achieved at the parts-per-trillion level if the soil does not contain charcoal. Recovery through the cleanup procedures exceeds 90% in all cases, being greater for samples containing lower levels of total extractable organic matter. The cleanup procedure allows processing of approximately 20-24 samples per week, a signficiant increase over previous methods. Tetra- through octachlorinated dioxins and furans are recovered to equivalent extents, which is

not the case for most alternative methods. (2) Means for processing samples of adipose tissue 600 times larger than any we have used previously had to be developed for the round-robin study. We were able to successfully prepare uniformly spiked samples with picogram quantities of analytes at known levels, but to obtain uniformity it was necessary to spike after extraction. It seems to be beyond the state of the art to guarantee spiking uniformity if attempts are made to add the analytes at this level to unprocessed adipose tissue. Analytical results from the participating laboratories have not yet been received (3) 2,3,7,8-TCDD bound to soil from Times Beach, Missouri, and from the Minker-Stout site in Missouri, was bioavailable upon oral ingestion by rats and guinea pigs. Analysis of the livers of the test animals indicated that soil-bound TCDD was absorbed and accumulated between 30-50% as efficiently as TCDD in corn oil. TCDD bound to a low-humus, sandy soil was more efficiently "extracted" and bioaccumulated than TCDD bound to Times Beach soil, with TCDD bound to red clay intermediate.

The extreme acute toxicity of some of the chlorinated dibenzo-p-dioxins and dibenzofurans (e.g. LD_{50} of 0.5 µg/Kg in the guinea pig for 2,3,7,8-TCDD and roughly 5 µg/Kg for 2,3,7,8-TCDF), combined with the present lack of understanding of the biochemical mechanism underlying the toxicity makes it necessary to perform biological experiments using extremely tiny amounts of material, often in the picogram and nanogram range. Dosing solutions involve amounts of material too small to weigh accurately, and determination of residual levels of test substances in tissues requires parts-per-trillion level analyses. Since these compounds are environmental pollutants, analytical results giving false negatives would contribute to a risk of poisoning, while false positives would have a major economic impact mandating extensive but unnecessary cleanup of the area sampled. In order to properly assess human health hazards from these compounds, among the fundamental data needed by various agencies are accurate and reliable measurements of the levels of dioxins and furans in the environment and in tissues of exposed subjects. Developing the means to obtain such data is a necessary precedent both to health hazard assessment and to well-controlled biomedical research. Further, both end-users must know how much confidence they can place in analytical data.

B. Plans for Subsequent Year

Since this project involves a response to expressed needs for development of methodologies, future work will depend upon what needs are expressed. There are no current plans for specific method development pending.

C. Publications of past 18 months

Parker, C.E., Albro, P.W., Bobenrieth, M.J., Cochran, T.W., and Robinson, J.D.: Quantitation of halogenated aromatic compounds by gas chromatography-mass spectrometry. J. Chromatogr. 278: 1-11, 1983.

McConnell, E.E., Lucier, G.W., Rumbaugh, R.C., Albro, P.W., Harvan, D.J., Hass, J.R., and Harris, M.W.: Dioxin in soil: Bioavailability after ingestion by rats and guinea pigs. Science 223: 1077-1079, 1984.

Albro, P.W., Lorenzo, J., and Schroeder, J.L.: Simultaneous monitoring of 3-H and 14-C and determination of the 3-H/14-C ratio in the effluent of a liquid chromatograph using solid scintillators. LC 2: 310-312, 1984.

Albro, P.W., Parker, C.E., Abusteit, E.O., Mester, T.C., Hass, J.R., sheldon, Y.S., and Corbin, F.T.: Determination of the pKa values of Metribuzin and its metabolites: A comparsion of spectrophotometric and potentiometric methods. J. Ag. Food Chem. 32: 212-217, 1984.

Tondeur, Y., Hass, J.R., Harvan, D.J., Albro, P.W., and McKinney, J.D.: Determination of suspected toxic impurities in Firemaster FF-1 and BP-6 by capillary column gas chromatography-high resolution mass spectrometry. J. Ag. Food Chem. 32: 406-410, 1984.

Tondeur, Y., Hass, J.R., Harvan, D.J., and Albro, P.W.: Computer-assisted determination of masses in high-resolution mass spectrometry by selected ion monitoring. Anal. Chem. 56: 373-376, 1984.

Albro, P.W., Parker, C.E., Marbury, G.D., and Hernandez, O.: Spectrometric characterization of Metribuzin and its metabolites. Appl. Spectroscopy (in press).

Tondeur, Y., Albro, P.W., Hass, J.R., Harvan, D.J., and Schroeder, J.: Matrix effect in determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin by mass spectro-

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30015-10 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies in Gaseous Ion Chemistry.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Ronald Hass	Research Chemist	LMB
OTHER:	Maurice M. Bursey	Visiting Scientist	LMB
	Earl White	Chemist	LMB
	Mike Kinter	Chemist	LMB
	Donald J. Harvan	Chemist	LMB
	Carol E. Parker	Chemist	LMB

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Mass Spectrometry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.2	4.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The unimolecular and collision-induced decompositions of $(NaF)2Na^+$ ions formed by excitation of a sodium fluoride surface with 7 keV Xe ion was investigated. By comparing the results with ab initio calculations, it was possible to demonstrate the presence of two structures of this cluster ion, differing in their internal energy content. The lower energy state (i.e., more stable) was similar to that of the crystal structure. By application of a tandem quadruple mass spectrometer system, the conversion of kinetic energy into internal energy was investigated for the molecular ion of 2-pentanone. It was found that between 14 and 25% of the available kinetic energy was converted into internal energy. Onset of conversion to internal energy through an electronically excited state was observed at 2.0 eV. In a second study, it was demonstrated that, at least for $[C_4R_4]^+$ ions, there exists a Hammett correlation for ratios of intensities of collision-induced fragments on the basis of the statistical theory of mass spectra and in practice. To help define the gas-phase reaction paths leading to the formation and subsequent decomposition of PO_3^-, the negative chemical ionization mass spectra of a group of dimethylvinyl and dimethylaryl phosphates were obtained, employing a variety of mass-spectral techniques. In every case, a major pathway to PO_3^- appears to consist of the sequence (a) capture of a thermal electron by the molecule, (b) loss of the vinyl or aryl group to produce the dimethyl phosphate anion, and (c) elimination of dimethyl either to yield PO_3^-. This ion, when suitably activated, decomposes further only by losing an oxygen atom to yield PO_2^-, the metaphosphite anion. The mechanism for the substitution reactions of NH_3 in the ammonia chemical ionization mass spectra of aromatic esters has been investigated. The formation of the NH_3 adduct ion involved the protonated molecule and the formation of an unstable intermediate which then underwent a reactive collision with another NH_3 to give the adduct product ion.

A. Studies in Gaseous Ion Chemistry

Nature of Problem: The general statement of the problem approached in this project is that the fundamental chemistry underlying one of our major analytical techniques is not well understood. Although a reasonable level of success has been achieved using an empirical basis, the rate of future progress will be dependent upon improvements in our understanding of the basic principles underlying our research. A secondary problem which falls within this project is the study of highly reactive intermedates whose existence is too transient to be observed in condensed phase media (i.e., solutions or biological systems).

Objectives: This project has two objectives. One is to improve our understanding of gas phase ion chemistry so that more powerful analytical methods, based upon this chemistry, can be developed and applied to environmental health problems. The second objective is the exploitation of the unique environment within the mass spectrometer to form and study the properties of highly reactive, but possibly thermodynamically stable, species that are of possible importance as intermediates in chemical and biological reactions.

Experimental Approach: The experimental approach used for questions in gas phase ion chemistry were a variety of ionization techniques including ion-molecule reactions and electron or particle bombardment of the material of interest. The products of this ionization process were characterized by mass spectrometry and tandem mass spectrometry with collisional activation being employed as required with the latter technique.

Recent Accomplishments: The recent accomplishments and their significance are discussed below for each study.

The unimolecular and collision-induced decompositions of $(NaF)_2Na^+$ ions formed by excitation of a sodium fluoride surface with 7 keV Xe ion was investigated as a means of increasing our understanding of the fundamental processes underlying the increasingly important analytical technique of particle beam indiced ionization (FAB or SIMS) and to validate the relationship between condensed phase and gaseous structure. By comparing the results with ab initio calculations, it was possible to demonstrate the presence of two structures of this cluster ion, differing in their internal energy content. The lower energy state (i.e., more stable) was similar to that of the crystal structure, except that bonds had undergone a considerable reduction in length during the volatilization process. At least for this simple system, particle beam ionization seems to retain the essential features of secondary structure from the condensed phase.

One of the critical areas in the application of tandem mass spectrometry to environmental health research problems is the excitation of gaseous ions by means of collisions with an inert neutral particle, typically rare gas atoms. Although routinely applied in analytical problems, this process is pooorly understood. By application of a tandem quadruple mass spectrometer system, the conversion of kinetic energy into internal energy was investigated for the molecular ion of 2-pentanone. It was found that between 14 and 25% of the available kinetic energy was converted into internal energy and hence into a form that can lead to fragmentation of the molecule. Onset of conversion to internal energy through an electronically excited state was observed at 2.0 eV. In a second

study, it was demonstrated that, at least for $[C_4R_4]^+$ ions, that there exists a Hammett correlation for the ratios of intensities of collision-induced fragments on the basis of the statistical theory of mass spectra and in practice.

Negative chemical ionization (NCI) mass spectrometry has proven a very useful analytical technique in environmental health sciences because of its selectivity towards electronegative compounds such as those containing halogen. An area that has received less attention is that of structural analysis beyound the normal limits of mass spectrometry, for example, the distinction of isomeric compounds. In this study, the possibility of using NCI followed by charge reversal mass spectrometry was explored. The study involved relatively simple ketones (so that ab initio molecular orbital calculations could be used) and the specific question was whether the stability of the negative ion formed by proton abstraction or the charge density (hence, acidity) of the hydrogen governed which proton is abstracted during hydroxide NCI. It was found that the dominant factor was the stability of the product ion. Following further studies to demonstrate the generality of this observation, it will be possible to incorporate this technique into problems requiring the identification of unknown compounds.

The monomeric metaphosphate anion, PO_3^-, has been widely proposed as an intermediate in the hydrolysis of phosphomonoesters (for example, ATP) in aqueous media. Our laboratory was the first to supply direct experimental proof that this anion not only exsits, but that it is thermodynamically quite stable. This first observation was in the NCI mass spectra of certain phosphotriesters. To help define the gas-phase reaction paths leading to PO_3^-, the NCI mass spectra of a group of dimethylvinyl and dimethylaryl phosphates were obtained, employing a variety of mass-spectral techniques. In every case, a (and perhaps, the) major pathway to PO_3^- appears to consist of the sequence (a) capture of a thermal electron by the molecule, (b) loss of the vinyl or aryl group to produce the dimethyl phosphate anion, and (c) elimination of dimethyl ether to yield PO_3^-. This ion, when suitably activated, decomposes further only by losing an oxygen atom to yield PO_2^-, the metaphosphite anion. In addition to the insights into the chemistry of a biologically important species, this study illustrates the power of mass spectrometry to isolate and study thermodynamically stable ions that are too reactive to be observed in condensed phase studies.

One of the more important uses of mass spectrometry is in the identification of chemical compounds. This traditionally involves the measurement of molecular weight and elemental formula followed by the interpretation of a fragmentation pattern. Although this is the most successful instrumental approach ever to be applied to such problems, the technique is frequently unable to provide sufficient information to permit the definitive assignment of structure, especially when a number of isomeric variants of a molecule are possible. The traditional approach has been the synthesis of reference compounds so that chromatographic parameters can be included in the identification criteria. This approach is valid, but laborious, and rarely applied properly. Before the widespread availability of analytical instrumentation, compounds were identified on the basis of their chemical reactivity. Toward the end of developing similar methods using gas phase reactions and the much higher sensitivity of mass spectrometry, we are investigating in some detail the reactivity of protonated molecular ions with ammonia. During this reporting period, we have demonstrated

that the mechanism for substitution reactions of NH_3 in the ammonia chemical ionization mass spectra of aromatic esters follows a straight-forward second order nucleophilic aromatic substitution reaction mechanism. The formation of the NH_3 adduct ion involved the protonated molecule and the formation of an unstable intermediate which then underwent a reactive collision with another NH_3 to give the adduct product ion. This mechanism is more complicated than originally thought.

The process of charge reversal is the formation of a positive ion by the removal of two electrons from a negative ion. The positive ions thus formed are generally in an unstable configuration. Thus this process is convenient for the study of highly reactive, unstable species that cannot be easily prepared otherwise. The study of these species broadens our knowledge of gaseous ion chemistry and hence improves our ability to apply mass spectrometry to analytical problems. In this particular study, the first work on gaseous nitrenium ions was reported. The methylnitrenium, ethylnitrenium and dimethylnitrenium ions were prepared by charge reversal collisional activation of the corresponding negative ions; their collisional activation mass spectra are shown to support the assigned structures. MINDO/3 energies are used to evaluate relative energies of $[CH_4N]^+$ and $[C_2H_6N]^+$ isomers, and to determine whether unstable forms rearrange spontaneously to stable ones. As in other examples, charge reversal here generates cations that do not exist in an energy well, but their transient existence is established because their fragmentation is more rapid than their rearrangement to a more stable form.

B. Plans for Subsequent Year

This project will be continued along present lines. Special emphasis is to be given the production of ions from surfaces and to the characterization of the collisional activation process. The former will be pursued through the study of the mass spectra of reference compounds with a highly focussed ion beam as the means of sample excitation; the latter by analogous studies involving the collisional activation of ions resulting from componds of known structure. The project exploring the reactivity of NH_3 in the gas phase will be continued. The work on PO_3^- seems to have reached a reasonable end-point. This project will not be continued.

C. Publications of past 18 months

Bursey, M.M. and Hass, J.R.: Correlation of acyl ion intensities with critical energies from collisional activation decomposition of small aliphatic ketones. Org. Mass Spectrom. (Submitted).

Bursey, M.M., Harvan, D.J., and Hass, J.R. Mass-analyzed ion kinetic energy study of flourine-substituted tetraphenyltetrahedrane ions. Org. Mass Spectrom. (Submitted).

Bursey, M.M., Harvan, D.J., Hass, J.R. and Becker, E.I.: Correlations between fragment ion intensity and critical energy for unimolecular bond dissociation. Org. Mass Spectrom. (Submitted).

Bursey, M.M. and Hass, J.R.: Study of collisional activation of CH_3S^+ from dimethyl sulfide with high precursor ion resolution. J. Am. Chem. Soc. (submitted).

Fraisse, D., Tabet, J.C. and Hass, J.R.: Ion-molecule reactions in the gas phase. V. Bimolecular aromatic substitution from substituted aromatic esters under ammonia chemical ionization. Org. Mass Spectrom. (submitted).

Bursey, M.M., Marbury, G.D. and Hass, J.R.: Fractionation of alkali metal cations in cationization of phthalic acid. A fast atom bombardment study. Biomed. Mass Spectrom. (submitted).

Meyerson, S., Harvan, D.J., Hass, J.R., Ramirez, F., and Maracek, J.F.: The monomeric metaphosphate anion in negative ion chemical ionization mass spectra of phosphotriesters. J. Am. Chem. Soc. (Submitted).

Bursey, M.M., Harvan, D.J., Parker, C.E., Darden, T., and Hass, J.R.: Charge reversal of three akylamide ions: Free nitrenium ions. Org. Mass Spectrom. 18: 530-533, 1983.

Hass, J.R. and Stoll, R.G.: Structure and fragmentation reactions of cluster ions produced by SIMS analysis of NaF. Org. Mass Spectrom. (submitted).

Nystrom, J.A., Bursey, M.M., and Hass, J.R.: Conversion of kinetic energy to internal energy in the 2-pentanone molecular ion at threshold in low-energy collisions with helium target atoms. Int. J. Mass Spectrom. Ion Proc. 55: 263-274, 1983/1984.

Hoppilliard, I., Baer, T., and Hass, J.R.: Experimental and theoretical structures and stabilities of gas phase alkyl ketone anions. Location of the acidic site. J. Am. Chem. Soc. (submitted).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30020-13 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Transport and Metabolism of Phthalate Esters

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Phillip W. Albro	Research Chemist	LMB	NIEHS
OTHER:	Richard Philpot	Research Chemist	LP	NIEHS
	J. Ronald Hass	Research Chemist	LMB	NIEHS
	Robert London	Research Physicist	LMB	NIEHS

COOPERATING UNITS *(if any)*

Gary Liss, M.D., CDC, NIOSH
P.K. Seth, Ph.D., Lucknow, India
B. Lake, Ph.D., B.I.B.R.A., UK

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.1	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The undesirable biological effects of phthalate esters (the most ubiquitous of all environmental pollutants), which include acute testicular atrophy resulting in male sterility, hepatocarcinogenesis in rats and mice, and proliferation of peroxisomes, are mediated through their metabolites. We have identified some 27 metabolites of the most widespread phthalate, di(2-ethylhexy)phthalate (DEHP), have postulated a metabolic pathway for their formation, have confirmed and detailed the first two steps in the pathway, and have formed an hypothesis as to the part of the pathway responsible for the dramatic differences in metabolic profiles in different species. As the biological activities of the different metabolites are elucidated, it becomes clear that species differences in metabolism can potentially result in resistance of non-rodent species to the undesirable biological effects. This makes extrapolation of toxicity tests from rodents to man highly unreliable, even though we have also provided evidence for general internalized exposure of humans to phthalate esters.

A. Transport and Metabolism of Phthalate Esters

Nature of Problem: When environmental chemicals exert undesirable biological effects directly, or when metabolism is required but is simple and straight forward, the question of the mechanism of action can be addressed in the laboratory by a variety of clearcut approaches. However, when environmental chemicals are ciated with significant biological effects, but the effects are mediated through a complex and ill-defined combination of metabolic alterations, it becomes impossible to delineate the mechanism(s) of action until the intermediary metabolism is understood in detail.

Objectives: (1) To define the role of metabolic alteration in the mechanism by which selected environmental chemicals exert their undesirable bioloigcal effects. Phthalate esters are used as model compounds because of the large number of microsomal, lysosomal, mitochondrial, peroxisomal, cytoplasmic and extracellular xenobiotic-metabolizing enzymes involved in their metabolism. (2) To elucidate the metabolic pathways involved in the catabolism of phthalate diesters and to identify the biologically active metabolites. (3) To elucidate the manner in which persistent environmental pollutants may alter the metabolic capabilities of various animal species, either through induction, activation, or inhibition. (4) To develop methodologies on which a data base related to human exposure can be built.

Experimental Approach: Metabolic pathways are traced using radioisotopically labeled chemicals, identifying metabolites through the use of high performance liquid and gas chromatography, mass spectrometry, infrared, ultraviolet and NMR spectroscopy, and chemical derivatization. In vivo studies involve a variety of laboratory animal species. In vitro studies involve primary hepatocyte tissue culture, liver slices, and microsomes prepared from rat and rabbit liver, lung and kidney. Compounds such as tetrachlorodibenzo-p-dioxin (TCDD), phenobarbital, clofibric acid, and PCBs are used as inducers of metabolizing enzymes. Standard techniques of enzyme kinetics, isolation of purified enzymes, isolation of DNA, and equilibrium binding techniques are used. Interactions of chemicals with enzymes are studied by fluroescence polarization, circular dichroism, equilibrium dialysis and reaction kinetics. Suspect compounds are tested for ability to bind covalently to macromolecules in target tissues. Ability to stimulate the release of active oxygen species is studied by incubating test chemicals with purified peroxisomes, in a linked assay involving coenzyme A activation of carboxylic acid metabolites. Peroxisome proliferation, induction of specific cytochromes, and in vitro metabolism are studied in primary hepatocyte cultures derived from rats, hamsters, and marmosets.

Recent Accomplishments and Significance: (1) 2-heptanone (a metabolite of di-(2-ethylhexyl)phthalate) or its metabolites appears to bind covalently to macromolecules, both protein and DNA, in rat liver in vivo. A variety of adducts result, but have not yet been identified. (2) Urinary phthalate levels correlate with exposure in human factory workers exposed to phthalate plasticizers and/or phthalic anhydride. However, phthalate is in human urine even in the absence of known work exposure. (3) The metabolism of phthalate ester by

liver microsomes apparently involves the P-450 dependent monooxygenases responsible for fatty acid omega and subterminal hydroxylation. One of the P-450 cytochromes involved is induced by clofibrate in rats, while another is induced by phenobarbital in rabbits but only slightly if at all in rats. Both monooxygenases are markedly suppressed by exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. (4) In contrast to the enzymes that hydrolyze phorbol esters in skin, the absence of which may account for the susceptability of mice but not rats to skin tumor promotion by phorbol esters, both rat and mouse skin contains phthalate ester hydrolase activity at comparable levels. (5) The DEHP metabolite thought to be responsible for peroxisome proliferation in rodents (2-ethyl-5-keto-hexyl phthalate) is a very minor metabolite in humans, but a major metabolite in rats and mice. Its immediate precursor, 2-ethyl-5-hydroxyhexyl phthalate, is a major product in both rats and humans. Failure of primates to efficiently oxidize the 5-hydroxyl group to a ketone in comparison to rats may involve differences in the alcohol dehydrogenase isoenzymes of the two species.

The compounds being studied, phthalate esters (PAEs), represent the class of environmental pollutants of most widespread occurrence in the general environment, matching PCBs in distribution and exceeding them in concentration.

Phthalate esters are known to cause liver cancer in both rats and mice, as well as acute testicular atrophy resulting in sterility in rats. In no case is the biochemical mechanism even close to being understood. The biological effects of the phthalate esters are clearly mediated by their metabolites, which must therefore be identified. Since the metabolic profiles are very different between rodents and man, the basis for the species differences must be understood before it will be possible to relate animal toxicity data to human health hazard assessment. Human exposure levels to phthalates are unknown, so methods to determine these are needed.

Studies of the production of the large number of metabolites of PAEs have revealed a considerable amount of new information on metabolic capabilities that apply, since many of the enzymes are found to be relatively nonspecific, to other classes of xenobiotics. Identification of the particular PAE metabolites responsible for the biological activities will have predictive value in terms of other compound classes serving as precursors for similar metabolic products.

Plans for Subsequent Year: (1) Metabolic studies will concentrate on enzyme systems operative beyond the level of the microsomal monooxygenases. Species differences in susceptability to the undesirable biological effects of PAEs are hypothesized to reflect either differences at the level of alcohol dehydrogenase or glucuronyl transferase specificity, and these two enzyme systems will be studied in rats, mice, hamsters, and marmosets. Isozyme comparisons and comparisons of substrate specificities will be performed. (2) The stage of spermatogenesis at which some unidentified metabolite of DEHP exerts its atrophic effect has been pinpointed at B.I.B.R.A. An attempt will be made to isolate and the DEHP metabolite(s) that is/are present in the target cells at (and before) that time. (3) While PAEs are causing testicular atrophy, they are also causing greatly increased urinary excration of zinc. The ability of individual

metabolites of DEHP to sequester or chelate zinc will be evaluated using NMR techniques. The possibility that the excess urinary zinc may be present as a chelate of DEHP metabolite I (2-ethyl-3-carboxypropyl phthalate) will also be investigated. Metabolite I is the major DEHP metabolite in urine of mice, and one of the three most abundant urinary metabolites in rats following a dose of DEHP sufficient to cause testicular atrophy.

C. Publications of past 18 months

Sherman, L., thompson, K., O'Kell, R.T., Albro, P., and Inksters, M.: Phthalate levels in microwave thawed fresh frozen plasma. Transfusion 22: 401, 1982.

Pohl, R.J., Serabjit-Singh, C.J., Slaughter, S.R., Albro, P.W., Fouts, J.R., and Philpot, R.M.: Hepatic microsomal NADPH-Cytochrome P-450 reductase from Little Scate, Raja erinacea. Comparison of thermolability and other molecular properties with a mammalian enzyme. Chem.-Biol. Interact. 45: 283-294, 1983.

Albro, P.W., Tondeur, Y., Marbury, D., Jordan, S., Schroeder, J., and Corbett, J.T.: Polar metabolites of di-(2-ethylhexyl) phthalate in the rat. Biochim. Biophys. Acta 760: 283-292, 1983.

Albro, P.W., Jordan, S., Corbett, J.T., and Schroeder, J.T.: Determination of total phthalate in urine by gas chromatography. Anal. Chem. 56: 247-250, 1984.

Albro, P.W., Corbett, J.T., Marbury, D., and Parker, C.: Urinary metabolites after oral administration of di-(5-hexenyl) phthalate and di-(9-decenyl) phthalate in the rat. Xenobiotica (in press).

Albro, P.W., Chae, K., Philpot, R., Corbett, J.T., Schroeder, J., and Jordan, S.: In vitro metabolism or Mono-2-ethylhexyl phthalate by microsomal enzymes. Similarity to ω- and (ω-1) oxidation of fatty acids. Drug Metab. Dispos. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30034-08 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemistry of Aromatic Compounds and Their Environmental Transformation Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Louis A. Levy	Research Chemist	LMB	NIEHS
OTHER:	A.R.K. Murthy	Visiting Fellow	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 10004-05 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30050-08 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical and Enzymatic Conjugation of Glutathione with Epoxides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Oscar Hernandez	Senior Staff Fellow	LMB	NIEHS
OTHER:	M.B. Gopinathan	Visiting Fellow	LMB	NIEHS

COOPERATING UNITS *(if any)*

Marine Pharmacology Section, LP, NIEHS and TRTP.

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-Organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One of the most important pathways available to organisms for the detoxication of potentially harmful chemicals is via reaction with glutathione. Among the numerous transformations involving alkylating reactions of glutathione, its reaction with epoxides was selected for thorough examination. This choice was dictated by our interest in the mechanism of biosynthesis of epoxides and the implication of these compounds as direct acting toxic agents. The project consisted of two phases, a chemical and an enzymatic component, for which the following objectives were defined: 1) develop methodology for the study of glutathione reactions with epoxides; 2) establish the stereochemical fate of these transformations; 3) elucidate the mechanism(s) of the enzyme catalyzed reaction of glutathione with epoxides. The first objective was accomplished with the development of methods which allow the preparation of diastereomerically pure glutathione conjugates as well as sensitive methods for the analysis of such compounds. The stereochemical course of the epoxide ring opening step was established with the aid of optically pure epoxides. This knowledge was translated into stereochemical profiles whereby diastereomer identification on a reversed-phase liquid chromatography system became possible. Experiments with various epoxide substrates and hepatic glutathione transferases from the little skate and the rat showed remarkable stereochemical consistency. In both cases, a majority of the purified enzymes showed a high preference for attack-addition of thiol at the R-carbon on the epoxide ring. Other enzymes showed the opposite preference, i.e. addition to the S-carbon. A mechanism was postulated to explain the stereoselectivity of this enzymatic transformation and based on this mechanism a model was proposed for the active site geometry of three glutathione transferases. The use of stereochemical profiles as a way of assesing differences, or similarities, among glutathione transferases is currently being explored.

A. Chemical and Enzymatic Conjugation of Glutathione and Epoxides

Nature of Problem: The metabolism of unsaturated hydrocarbons by the cytochrome P-450 monooxygenase system results in the formation of epoxides as the primary metabolites. Epoxides have been shown to be responsible for the toxic effects associated with exposure to unsaturated hydrocarbons, notably the polynuclear aromatic hydrocarbons. Consequently, it becomes important to understand the factors which may affect the metabolism and disposition of epoxides. One of the major pathways by which epoxides are detoxified is via conjugation with glutathione. This transformation is catalyzed by a group of enzymes collectively known as the glutathione transferases. At the outset of this project the mechanistic details available on this enzymatic transformation were insufficient to assess the extent of participation of the glutathione transferases in the regulation of epoxide metabolism.

Objectives: The initial objective was to establish the chemical mechanism of the enzyme catalyzed reaction of glutathione with epoxides. From mechanistic studies on model thiol nucleophiles, and kinetic information on some glutathione transferases, a picture emerged suggesting that the most likely pathway for thiol addition to the epoxide ring was via a borderline S_N2 mechanism. Experiments were designed to test this hypothesis using purified glutathione transferases and epoxide substrates for which chemical information had been made available through parallel studies.

Experimental Approach: Our experimental design was based on the observation that the ratio of diastereoisomers formed in the reaction of glutathione and a racemic epoxide was drastically different when subjected to enzymatic catalysis. Thus it became possible to utilize a high-performance liquid chromatography assay for the estimation of diastereoisomers generated by the different ransferases. The information gathered, kinetic as well as stereochemical, would be eventually integrated to formulate a mechanism for the enzymatic step. An additional benefit of this stereochemical approach was that, for the first time, it would be possible to quantitate the extent of participation of the glutathione transferases in the conjugation of epoxides.

Recent Accomplishments and Significance: The mechanistic aspects of the glutathione transferase reaction underscore the relevance of sterochemical factors in influencing the rate of elimination of enantiomeric oxides via the glutathione pathway.

C. Publications of past 18 months

Hernandez, O. and Bend, V.R.: Metabolism of Epoxides. In Jakoby, W.B., Bend, J.R. and Caldwell J. (Ed.): Metabolic Basis of Detoxication. New York, Academic Press, Inc., 1982, pp. 207-228.

Pagano, D.A., Yagen, B., Hernandez, O., Bend, J.R. and Zeiger, E.: Mutagenicity of (R)- and (S)-styrene 7,8-oxide enantiomers and the intermediary mercapturic acid metabolites formed from styrene 7,8-oxide. Environ. Mutagen. 4: 575-584, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30051-08 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Specific Binding Toxic Polyhalogenated Aromatic Hydrocarbons

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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OTHER:	Philip W. Albro	Research Chemist	LMB	NIEHS
	Kun Chae	Chemist	LMB	NIEHS
	E.E. McConnell	Veterinary Pathologist	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 30066-08 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 30064-07 LMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development of Analytical Methodology for Environmental Health Sciences.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	Reiner G. Stoll	Expert	LMB
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COOPERATING UNITS *(if any)*

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SECTION
Mass Spectrometry

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	3.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new ion source, using negative primary ions, for liquid secondary ion mass spectrometry (LSIMS) was designed and constructed. The most significant advantages were that 10 kV power supplies (compared to 20 kV for positive sources) were adequate, resulting in simpler design requirements and reduced electrical arcing in the source. A highly focused point source was fitted to a double focusing mass spectrometer which was also equipped with a moveable target. An ion optical system was designed and constructed so that secondary ions formed on a flat surface could be extracted and injected into the mass spectrometer. Intense secondary ion currents (~10^{-11} to 10^{-12} amps) were obtained from organic compounds, despite the power dissipation of ~10^{+4} watts per cm^2. By use of a piezoelectric-effect crystal motor system and microcomputer, it was possible to move the sample reproducibly in 10^{-8} m steps (10 nm) in two directions. Surface distributions of organic chemicals could be obtained with a spatial resolution <1 micron. The idea of chloride attachment negative chemical ionization (NCI) mass spectrometry was extended to HPLC/MS. The world's first tandem high resolution mass spectrometer was built to our specifications and installed. This instrument permits simultaneous high mass resolution on both parent and daughter ions. Of perhaps greater environmental health interest, moderate resolutions can be achieved on both parents and daughters at good sensitivity. Initial studies have demonstrated the power of this instrument for the characterization of peptides, corticosterones and phospholipids from biological sources. Advantages include greatly increased analytical information and a much reduced susceptibility to sample contamintion. In addition to the new tandem instrument, the use of kinetic energy release (KER) measurements has been extended from its traditional role in the study of gaseous ion chemistry to the characterization of unknown organic compounds. A new time-of-flight design has been developed which offers resolution equal to or better than that achieved when magnetic instruments are operated at very high mass (i.e.>8000 daltons).

A. Development of Analytical Methodology for Environmental Health Sciences
Nature of Problem: The problem addressed in this research project is the limitations imposed upon environmental health research by the analytical methods available for the identification and/or determination of the compounds of interest. In this project, our attention is restricted to the enhancement of mass spectrometry to permit new areas of environmental health questions to be addressed.

The objectives of this project are to reduce the particular limitations set by sample volatility requirements, sample purity requirements and the relatively low molecular weights accessible with present instruments.

Objectives: The experimental approach to this project involves the extension of mass spectrometry so as to permit either new problems to be addressed or more definitive answers to be given in areas of interest in environmental health sciences. Work has concentrated in three areas: ion source technology, tandem mass spectrometry (TMS) and combined high performance liquid chromatography/mass spectrometry (HPLC/MS). In addition, a new time-of-flight (TOF) mass analyzer has been designed for the measurement of molecular weights of polar molecules in the range up to 10000-15000 daltons.

Recent Accomplishment: The recent accomplishments and their significance are summarized below for each project.

Ion Source Technology:

A new ion source for liquid secondary ion mass spectrometry (LSIMS) was designed and constructed. This ion source is unique in that it permits the use of negatively charged primary ions so that higher impact energies could be attained at the sample surface. According to the most popular model for LSIMS, the secondary ion yield should increase with primary ion energy under these conditions. This source was found to give only marginally improved performance when compared to sources utilizing positive ions on the same mass spectrometer. The most significant advantage was that 10 kV power supplies (compared to 20 kV for positive sources) were adequate resulting in simpler design requirements and reduced electrical arcing in the source.

A highly focused point source was fitted to a double focusing mass spectrometer which was also equipped with a moveable target. An ion optical system was designed and constructed so that secondary ions formed on a flat surface could be extracted and injected into the mass spectrometer. Intense secondary ion currents ($\sim 10^{-11}$ to 10^{-12} amps) were obtained from organic compounds despite the power dissipation of $\sim 10^{+4}$ watts per cm^2. By use of a piezoelectric-effect crystal motor system and a micro computer, it was possible to move the sample reproducibly in 10^{-8} m steps (10 nm) in two directions. Tuning the mass spectrometer to transmit an ion indicative of a given molecular species on a surface permitted surface distributions to be mapped. The coating of suitable

organic materials on fine mesh screen wires permitted the demonstration of spatial resolutions of less than < 1 micron. Early attempts to map chemicals in a tissue sample failed. Work is currently in progress to characterize the ion formation mechanism so that complex biological samples may be analyzed at high spatial resolution.

The idea of chloride attachment negative chemical ionization (NCI) was extended to HPLC/MS. This was accomplished by the addition of a small quantity of chlorinated solvent modifier to the HPLC solvent system. Temperature studies indicated that the same ionization mechanisms apply in the HPLC case as in the earlier direct probe analyses. By including the HPLC/MS, the chloride attachment NCI technique can be extended to the analysis of mixtures. A new mass spectrometer has been acquired that includes a thermospray interface. Studies are underway to determine the applicability of this technique to problems in environmental health sciences.

The major development in our tandem mass spectrometry project has been the delivery of the world's first tandem high resolution mass spectrometer which was built to out specifications. This instrument permits, if necessary, simultaneous high mass resolution on both parent and daughter ions and thus represents the most powerful tandem mass spectrometer ever constructed. Of perhaps greater environmental health interest, moderate resolutions can be achieved on both parents and daughters at good sensitivity. Initial studies have demonstrated the power of this instrument for the characterization of peptides, steroids and phospholipids from biological sources. Advantages include greatly increased analytical information and a much reduced susceptibility to sample contamination. Further studies are in progress to determine the scope and limitations of this instrument in these and other areas of interest in environmental health sciences. In addition to the new tandem instrument, the use of kinetic energy release (KER) measurements has been extended from its traditional role in the study of gaseous ion chemistry to the characterization of unknown organic compounds. In particular, it was found that the KER was adequate to distinguish aliphatic and aromatic carbon-halide bonds for chlorinated and brominated compounds. This distinction is difficult or impossible otherwise when compounds which can contain either functionality are present in complex mixtures.

Recent developments in ion source technology make it reasonable to attempt the measurement of molecular weights in range up to 10000-15000 daltons. The commercial instruments being developed to take advantage of this progress involve even larger (and hence, more expensive) magnetic mass spectrometers. An alternative, which is much less expensive and simpler, is to use the time-of-flight (TOF) principle for mass analysis. An additional advantage is that TOF instruments have very high transmission and hence should be more sensitive than magnetic instruments. The major limitation has been mass resolution. In the past year, we have developed a new TOF design which offers resolution equal to or better than that achieved when magnetic instruments are operated at very high mass (i.e. >8000 daltons).

B. Plans for the Subseqent Year:

Major emphasis will be placed on the characterization of the properties of the

tandem double-focusing mass spectrometer. This will include development of algorithms to permit mass calibration under a full range of conditions as well as exploring the new experimental possibilities now open (charge reversal at high daughter ion mass resolution, ionization within the collision cell, etc.). The next task with the focussed ion source project is to characterize ion yields under conditions approximating those of biological samples and to develop sample handling techniques so that surface morphology work can be undertaken. As discussed above, the ultra-high mass range mass spectrometer project awaits only available manpower resources for its initial construction. Should these be made available, this instrument will be constructed and put to immediate use characterizing peptides and other biological molecules in the molecular weight range up to 10000-20000.

C. Publications of past 18 months:

Tondeur, Y., Albro, P.W., Hass, J.R., Harvan, D.J., and Schroeder, J.L.: Matrix effects and sub-part-per-billion quantitative analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin by mass spectrometry. Anal. Chem. (in press).

Tondeur, Y., Hass, J.R., Harvan, D.J. and Albro, P.W.: Computer assisted determination of masses in high resolution mass spectrometry with selected ion monitoring. Anal. Chem. 56: 373-376, 1984.

Voyksner, R.D., Hass, J.R. and Bursey, M.M.: Correlation of kinetic energy release with the structure of selected chlorinated compounds in mass-analyzed ion kinetic energy spectrometry. Anal. Chem. 55: 915-920, 1983.

Voyksner, R.D., Sovocool, G.W., Bursey, M.M. and Hass, J.R.: The comparison of GC/MS with MS/MS in the analysis of polychlorinated biphenyls and tetrachlorobibenzofurans. Anal. Chem. 55: 744-749, 1983.

Hass, J.R., Tondeur, Y. and Voyksner, R.D.: The use of kinetic energy release to differentiate between brominated compounds. Anal. Chem. 55: 295-297, 1983.

Parker, C.E., Albro, P.W., Abusteit, E.O., Mester, T.C., Hass, J.R., Sheldon, Y.S., and Corbin, F.T.: Determination of the pKa values of metribuzin and its metabolites: A comparison of spectrophotometric and titrimetric methods. J. Ag. Food Chem. (in press).

Voyksner, R.D., Hass, J.R. and Bursey, M.M.: Isomer identification by kinetic energy release measurements using the voltage pulsed collision cell technique in mass analyzed ion kinetic energy spectrometry. Biomed. Mass Spectrom. (in press).

Stoll, R.G., Harvan, D.J. and Hass, J.R.: A focussed SIMS source for the analysis of organic compounds. Int. J. Mass Spectrom. Ion Proc. (in press).

McEwen, C.N. and Hass, J.R.: An Au- ion gun for liquid secondary ion mass spectrometry. Org. Mass Spectrom. (submitted).

Hass, J.R., Green, B.N., Bott, P.A., and Bateman, R.D.: Design and performance of a tandem double focussing mass spectrometer. Int. J. Mass Spectrom. Ion Proc. (submitted).

Hass, J.R., Green, B.N. and Bordoli, R.S.: The application of a tandem double-focussing mass spectrometer to the characterization of peptides. J. Am. Chem. Soc. (submitted).

Parker, C.E. and Hass, J.R.: High performance liquid chromatography in combination with chloride attachment negative chemical ionization mass spectrometry. J. Chromatog. (submitted).

Walther, H.J., Hass, J.R. and Marbury, G.D.: Deoxy-dinucleotides by fast atom bombardment mass spectrometry and linked scan MS/MS. Biomed. Mass Spectrom. (submitted).

Bursey, M.M., Hass, J.R., Sullivan, B.P. and Meyer, T.J.: Extension of fast atom bombardment to mass spectrometry of an inefficiently ionized organometallic complex by double focussing before collisional activation. Anal. Chem (submitted).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30065-07 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Mass Spectral Reactions in Field-Free Regions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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OTHER:	Carol E. Parker	Chemist	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Mass Spectrometry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 30064-07 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 30066-08 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Theoretical Basis and Molecular Mechanisms of Biological Action

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-Organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Halogenated aromatic hydrocarbons constitute a broad class of compounds with varying structure and toxicity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the prototypical structure, is the most toxic compound of this type. Theoretical, quantitative structure activity relationship (QSAR) and experimental studies focusing on polychlorinated biphenyls (PCBs) as models have established the importance of molecular polarizability and receptor to PCB separation distance in binding to the dioxin or Ah receptor. The work suggests a new stacking type model for the Ah receptor which has universal applicability to the range of binding-structures observed. Further theoretical and experimental studies suggested that Ah receptor binding is a necessary but not sufficient condition for toxicity and a second receptor must be proposed to account for the structural requirements of planarity and halogenation in toxicity, as opposed to induction. A protein binding model consistent with the structural requirements for toxicity has been identified. Molecular modeling work, competitive binding assays and several in vivo studies have demonstrated the importance of thyroxine binding proteins in toxicity and suggested that the high toxicity of TCDD is the expression of potent and persistent thyroid hormone activity. A molecular mechanism is proposed involving a two protein-receptor model in which the planar aromatic system controls the initial receptor binding and halogen substitutents control subsequent nuclear events. This mechanistic hypothesis is being further investigated for its relevance in dioxin toxicity as well as insight into the mechanism of thyroid hormone action.

A. Structural Theoretical Basis and Molecular Mechanisms of Biological Action of Aromatic Hydrocarbons: Dioxin and Related Compound Toxicity.

Nature of Problem: The halogenated aromatic hydrocarbons are a class of chemicals to which essentially everyone is chronically exposed at low levels and to which some people are occupationally exposed at relatively high levels. Compounds studied include members of the biphenyl (PCBs, PBBs) naphthalene (PCNs, PBNs), dibenzo-p-dioxin (CDDs) and dibenzofuran (CDFs) chemical classes. Among these classes are some of the most toxic small molecules known. Dermatological effects, including chloracne, and an increase in liver enzyme activity have been clearly demonstrated in human populations. However, health effects in the areas of hepatotoxicity, mutagenesis, and reproductive effects are still the object of a continuing controversy. Since our laboratory has been interested in the chemistry and biochemical toxicology of these compounds for many years and has developed many techniques used in their study, we decided to focus our attention on the halogenated aromatic hydrocarbons as model compounds for accomplishing research to meet our overall program objectives. Specifically we hope to determine the structural basis of dioxin and related compound toxicity and compare it with the same observed for other kinds of biological activity such as mixed function oxidase induction. This information in turn should be useful in assessing toxicologically relevant protein receptors in biological systems which could be further studied to delineate their role in the mechanims of toxicity. In the course of this work, we will develop our research hypotheses and conclusions in terms of structure and theoretical chemistry which should provide a basis for further studies in predictive toxicology.

Objectives: Develop and evaluate the use of structure-activity relationships in understanding molecular mechanisms of biological action for predicting the toxicological outcome. Develop systematically physical-organic measurements and calculations that charactize the structural properties important in these actions and explain how chemical structures determine the toxicological outcome. Use halogenated aromatic hydrocarbons as prototype compounds for these purposes.

Research Design: Specific isomers and congeners within these compound classes were synthesized or obtained from other sources for toxicological testing. Compounds are administered to guinea pigs as an exquisitely sensitive animal model to the toxic effects of these compounds. Representative compounds were synthesized or obtained radiolabeled to study their pharmacokinetic behavior in the guinea pig. Where needed some compounds were administered to rats to investigate their potential to induce cytochrome P450, P448 and related enzyme activities or alter other endogenous compounds. Experimental methods were devised to measure molar polarizability of biphenyls as a representative class. Ab initio quantum mechanical and semi-empirical calculations have also been devised to assess conformational and polarizability properties. Variety of chromatographic, spectroscopic and crystallographic techniques are used to isolate and characterize the compounds and provide definitive structural information.

After determining the structural basis of dioxin and related compound toxicity we hope to use the information to redirect efforts to isolate and identify toxicologically relevant receptor proteins. A variety of biochemical methods will

be used to establish the importance of identified receptor proteins in the mechanism of toxicity. Molecular graphics and computational chemistry are used to examine structural similarity and complementarity and redirect synthetic work as well as to examine the "quality" of fit and detailed atomic environment of toxic structure interactions with known protein binding sites.

Recent Accomplishments: The polychlorinated biphenyls (PCBs) have been studied as a prototype subclass of halogenated aromatic hydrocarbons that have "dioxin type" toxicity. The stereochemical nature of these compounds plays an important role in defining their toxicological properties. We have examined the conformation of PCBs using the Gaussian-80 ab initio technique. All molecules that do not have ortho-substitution have an energy minimum at 42° and barriers of approx. 3.6 and 2.3 kcal mol^{-1} occur at 0° (and 180°) and 90° respectively. Ortho-substitution results in an extremely high barrier at 0° (and 180°) and a shift of the energy minimum toward 90°. These results are consistent with the diminished toxicity of ortho-substituted PCBs if planarity of structure is important in their mechanism of action.

There are circumstances under which polarizability may be the determining factor in governing the interaction of two molecules at long range. This appears to be the case for the halogenated aromatic hydrocarbons studied in this work where permanent dipoles are weak and similar across the classes and where ionization energies are nearly identical throughout. The Applequist-Carl-Fung method for calculating polarizabilities of polyatomic molecules was modified for treating planar aromatic molecules including PCBs (planar and rotated), dioxins and polynuclear aromatic hydrocarbons. The essential modification was to allow for anisotropic carbon atom polarizabilities; the resulting molecular anisotropics were much improved. In addition, a binding model was proposed which approximately accounts for the polarizability contributions (through dispersion) to the relative binding constants of similar molecules to a common receptor and emphasizes the importance of a planar structure for strong binding.

A theoretical model based on the dispersion interactions discussed above was developed to interpret the cytosol-PCB binding data reported in the literature. The essential parameters in the model were the PCB polarizability and the receptor to PCB separation distance. An approximate linear free energy relationship was derived in terms of these parameters. It was suggested that the oxygen in dioxins may lead to stabilizing hydrogen bonding with the receptor; this reasonably accounts for the unusual binding affinity of certain dioxins. Finally, a derivation of $[EC_{50}]$ for the PCBs displacement reaction based solely on equilibrium theory was shown to lead to an expression consistent with the experimental correlation if the displaced TCDD aggregates with the solution PCB or any other cytosol component. This work suggests a new stacking type model for the dioxin or Ah receptor which has universal applicability to the range of binding structures.

These structural and theoretical studies have led us to compare the structure-induction (involving rat and mouse Ah receptor binding) and structure-toxicity (in vivo guinea pig toxicity) relationships. This comparison suggested that two receptors with structurally distinct binding properties may be involved. This

is supported by demonstration of potentiated toxicity through a mechanism believed to involve the Ah receptor as a site of loss with respect to toxicity. Theoretical and working models were proposed for these separate receptors to aid in the search for other relevant binding proteins which meet the requirements of planarity and halogenation for toxicity, as opposed to induction.

We have been seeking protein binding models consistent with the requirements of planarity and halogenation for toxicity. In molecular modeling work (using interactive computer graphics) involving thyroid hormones and thyroxine binding prealbumin, we directly and logically linked the structure of TCDD to the structure of thyroxine (T_4) and demonstrated that such structures can be competitive binding ligands for T_4 binding sites. Several TCDD studies including rat serum T_4 depletion as a function of dose, rat tissue T_4 depletion and effects on nuclear thyroid hormone receptor binding of T_4 and on tadpole metamorphosis support the T_4 agonist activity of TCDD in vivo and suggest that the high toxicity of this environmentally important compound is the expression of potent and persistent thyroid hormone activity. A cooperative receptor mechanism involving the Ah receptor is indicated. In support of the Ah receptor being a necessary but not sufficient receptor for toxicity, we have recently demonstrated that thyroid hormones can interact with the Ah receptor and that both T_3 and T_4 can potentiate the teratogenicity of TCDD, a toxic effect believed to involve the Ah receptor.

B. Plans for Subsequent Year

We hope to develop an improved computational method for polarizability that will permit us to estimate the relative binding to a common receptor of both non-symmetrical as well as symmetrical structures. This could enable predictions to be made about binding affinity across compound classes. Our goal is to estimate relative binding properties and thus relative toxicities for systems in which the polarizability interaction may be dominant. Using computer molecular graphics we will further screen selected halogenated aromatic hydrocarbons by examining the "quality" of fit to prealbumin as a possible predictor of their ability to interact with the putative "toxic" receptor. This is important since it is known that planarity of structure (and the associated polarizability interaction) and persistence in tissue are not the only controlling factors in the toxicity. An attempt will be made to combine the theoretical model for the Ah receptor with the prealbumin interaction model for the "toxic" receptor for predicting toxicity of this type across compound classes. We will also attempt to develop an experimental cooperative binding assay for this purpose combining the two protein binding assays into one.

Further work will be done to establish that these structures are thyroid hormone agonists (and possibly antagonists). For example, the ability to present a planar face also appears to be important in the expression of thyroid hormone activity. We will investigate this using biphenyl analogs of thyroid hormones and develop similar theoretical models to explain their binding properties. The involvement of nuclear thyroid hormone receptors in TCDD toxicity will also be studied in further establishing the relationship of the mechanism of dioxin and related compound toxicity to the mechanism of thyroid hormone action.

C. Publications of past 18 months

McKinney, J.D., Gottschalk, K.E. and Pedersen, L.: A theoretical investigation of the conformation of polychlorinated biphenyls. J. Mol. Structure 104: 445-450, 1983.

McKinney, J.D., Gottschalk, K.E. and Pedersen, L.: Polarizability of planar aromatic systems. An application to polychlorinated biphenyls, dioxins, and polyaromatic hydrocarbons. J. Mol. Structure. 105: 427-438, 1983.

McKinney, J.D., Long, G.A. and Pedersen, L.: PCB and dioxin binding to cytosol receptors: a theoretical model based on molecular parameters. QSAR in Pharmacology, Chemistry, and Biology, in press.

McKinney, J.D., Chae, K., McConnell, E.E. and Birnbaum, L.S.: Structureinduction versus structure-toxicity relationships for polychlorinated biphenyls and related compounds. Environmental Health Perspectives, in press.

Birnbaum, L.S., Weber, H., Harris, M.W., Lamb, J.C. and McKinney, J.D.: Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin: Increased incidence of cleft palate in mice. Toxicol. Appl. Pharmacol. submitted.

McKinney, J.D.: Molecular basis of chemical toxicity. Environmental Health Perspectives, in press.

Lamb, J.C., Harris, M.W., Weber, H., Mckinney, J.D. and Birnbaum, L.: Potentiation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced cleft palate (CP) by triiodothyronine (T_3) in C57BL/6N(B6) mice. Biol. Reproduction, in press.

Birnbaum, L.S., Darcey, D.J. and McKinney, James, D.: Hexabromonaphthalene contaminants of polybrominated biphenyls: Chemical composition and disposition in the rat. J. Toxicol. Environ. Hlth. 12: 555-573, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50046-06 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Chemically Induced Photosensitivity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Molecular Biophysics

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.1	4.1	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Light is known to interact with endogenous or exogenous chemical agents in the skin or eyes, to produce photosensitization (phototoxicity or photoallergy). While the initial step in all forms of photosensitivity must be the absorption of light by the chemical or its metabolites, the precise mechanisms of sensitization are unknown. The objective of this study is to determine whether light-induced free radicals or active oxygen species play a role in photosensitization. Benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benzoxazole acetic acid] is an anti-inflammatory drug that causes acute phototoxicity in many patients. Irradiation of benoxaprofen in anerobic organic solvents provided evidence for hydrogen abstraction reactions and a drug-derived carbon centered radical. In the presence of oxygen both superoxide and singlet oxygen were detected. Oxygen markedly increased the photohemolysis of human red blood cells by benoxaprofen suggesting that oxygen-derived species were involved. Inhibition of photohemolysis by the anti-oxidant butylated hydroxyanisole emphasized the importance of free radicals in this process. Anthracene, a component of coal tar, is a potent photosensitizing agent both in vivo and in vitro. Irradiation of anthracene in aerated ethanol produced singlet oxygen. Anthracene caused a concentration dependent photohemolysis of human erythrocytes that was markedly enhanced by the presence of oxygen and was abolished by β-carotene, a singlet oxygen quencher. These findings are consistent with the generation of singlet oxygen by irradiated anthracene, which in turn reacts with membrane lipids to produce peroxides that are responsible for membrane damage and, ultimately, hemolysis. Irradiation of the following chemical agents in aqueous or organic solvents also produced free radicals and/or active oxygen species: benzoxazole, 2-methylbenzoxazole, 2-phenylbenzoxazole, musk ambrette, chlorpromazine, furosemide and fluoranthene. These photoinduced species may play an important role in the phototoxic and photoallergic properties of these agents.

PROJECT DESCRIPTION

A. Mechanisms of Chemically Induced Photosensitivity

Nature of Problem: Light is known to interact with chemical agents in exposed tissues, such as the skin or eyes, to produce photosensitization. The chemical agent may be endogenous (protoporphyrin in erythropoietic protoporphyria), a drug (sulfonamides, declomycin, chlorpromazine), a topical agent (4-aminobenzoic acid and its esters in sunscreens; halogenated salicylanilides in soaps) or an environmental agent (polycyclic aromatic hydrocarbons in coal tar; amyl esters of 2-aminobenzoic acid in printer's ink). The photosensitivity response may be one of two types, phototoxic or photoallergic. The phototoxic reaction generally occurs during a subject's first exposure to sunlight, after the administration or topical application of a chemical or drug, and usually takes the form of an exaggerated erythemal response ("sunburn"). Photoallergic individuals may also exhibit an initial erythemal reaction. As this subsides, delayed abnormal responses may begin to appear including papular, eczematous and urticarial reactions. Such reactions to light may persist for months after avoidance of the photoallergen.

While the initial step in all forms of photosensitivity must be the absorption of light by the chemical or its metabolites, the precise mechanisms of photoxicity and photoallergy are unknown. The close similarity between phototoxicity and the normal erythemal response to excessive sunlight exposure suggests that the mechanisms are probably the same or very similar. In photoallergy, the agent is converted to a chemically reactive species (hapten) which then combines with a protein or other macromolecule (carrier) to form an allergen. The mechanism of the subsequent immunologic response is presumably similar to other types of delayed hypersensitivity.

When the ground state (S_0) of a molecule absorbs a photon of light it is converted to a very short-lived singlet excited state (^1S) which rapidly goes to a long-lived excited triplet state (^3S). Most photosensitized reactions in biological systems are mediated by the triplet state of the sensitizer. Subsequent reactions can proceed by a number of different pathways, depending on the chemical nature of the sensitizer and the substrate as well as the reaction conditions. Two major types of processes may occur, termed Type I and Type II. In Type I (free radical or redox reactions) reactions the triplet sensitizer molecule may abstract an electron (or hydrogen atom) from the substrate molecule (R) to give a semi-reduced (free radical) form of the sensitizer ($S^{\cdot}$) and a semi-oxidized (free radical) form of the substrate ($R^{\cdot}$). These processes may be summarized as follows:

$$S_0 \longrightarrow {}^1S$$

$${}^1S \longrightarrow {}^3S$$

$${}^3S + R \longrightarrow S^{\cdot -} + R^{\cdot +}$$

The semi-reduced form of the sensitizer may also react directly with oxygen to

give the superoxide radical ($O_2^{\cdot -}$)

$$S^{\cdot -} + O_2 \longrightarrow S + O_2^{\cdot -}$$

In Type II (energy transfer) reactions, electronic excitation energy is transfered from the triplet state sensitizer to ground state oxygen to give a highly electrophilic excited singlet state ($^1O_2$) of oxygen.

$$^3S + {}^3O_2 \longrightarrow S_0 + {}^1O_2$$

This form of oxygen reacts with many kinds of biomolecules much more rapidly than ground state oxygen.

Objectives: The objectives of this research are to determine the precise mechanisms whereby chemical agents produce photosensitization. This is achieved by first identifying those reactive species (free radicals, superoxide anion, singlet oxygen) that are generated when the photosensitizer is irradiated with light. This is then followed by a detailed study of the chemistry of the reactive species under conditions that are as close to physiological as possible. Finally, direct evidence is sought for the involvement of the reactive species that have been identified in the known biological response to a given photosensitizer. The biological studies are initially carried out with simple systems such as liposomes or the human erythrocyte. This work is then extended to more complex systems such as cells grown in culture or isolated from skin.

Experimental Approach: Free radicals may be detected, identified and quantitated by electron spin resonance (ESR) spectroscopy. However, since most free radicals generated photolytically are chemically reactive they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from the ESR spectrum of the resultant spin adduct.

Singlet oxygen may also be detected by ESR. One technique involves the use of a hindered amine (2,2,6,6-tetramethylpiperidine) which upon reaction with singlet oxygen yields a stable nitroxide (2,2,6,6-tetramethylpiperidine-1-oxyl). A second procedure involves the measurement of oxygen consumption by monitoring the effect on the linewidth of an exogenously added nitroxide. In both cases the presence of singlet oxygen is confirmed by the use of specific quenchers such as dibenzofuran, dimethylfuran and DABCO.

Recent Accomplishments

Benoxaprofen: Benoxaprofen [2-(4-chlorophenyl)-α-methyl-5-benzoxazole acetic acid] is an anti-inflammatory drug that causes acute phototoxicity in many patients. The photochemistry of benzoxazole and a series of structurally related analogs has been studied in aqueous and organic solvents. When benzoxazole or 2-methylbenzoxazole were irradiated in aqueous solution they underwent photo-ionization and photohomolysis. In the presence of oxygen, the superoxide radical was detected.

Irradiation of anerobic ethanolic solutions of benoxaprofen or 2-phenylbenzoxazole resulted in hydrogen abstraction from the solvent to yield hydroxyethyl and ethoxyl radicals. In the presence of suitable electron donors, eg. NaN_3, NaOH, these compounds acted as "shuttles" and transferred the abstracted electron to an appropriate acceptor. When oxygen was present, electronic energy transfer took place to give singlet oxygen, a powerful oxidizing agent.

These findings show that benzoxazoles are photochemically active and that they are able to participate in either Type I or Type II processes depending on the type of available substrate and the presence of oxygen.

Investigation of the excited-state chemistry of benoxaprofen in biphasic media (e.g., micelles and cyclodextrins) above and below the pKa of benoxaprofen has indicated that the neutral molecule is stable to photoexcitation, while the ionized form is photoreactive. Under certain conditions, the neutral benoxaprofen forms aggregates which display excimer-type fluorescence. Such interactions may play a role in the toxicity of the drug.

The possible in vivo importance of both oxygen-dependent and oxygen-independent processes has been demonstrated in studies of the light-induced benoxaprofen-dependent hemolysis of human erythrocytes. In this system the presence of oxygen markedly increased the rate of photohemolysis. Unfortunately, it proved impossible to determine unequivocally whether singlet oxygen and/or superoxide were involved in this process. However, inhibition of photohemolysis by the anti-oxidant butylated hydroxyanisole provided good evidence for the participation of free radicals in photohemolysis. Furthermore, irradiation of mast cells in the presence of benoxaprofen caused degranulation and the release of histamine. This may explain the urticarial response to benoxaprofen and light seen in human subjects.

Chlorpromazine:Chlorpromazine [2-chloro-N-(3-dimethylaminopropyl)phenothiazine] is a frequently prescribed antipsychotic drug which also causes phototoxic and photoallergic reactions. Irradiation of chlorpromazine is known to produce the chlorpromazine cation radical, as well as other photoproducts which may go through radical intermediates. As a preliminary to studying the radical photoproducts of chlorpromazine, the high-resolution ESR spectra of the chemically generated aqueous cation radicals of six phenothiazine derivatives were obtained and fully analyzed.

Under UV irradiation the radical resulting from dechlorination of chlorpromazine was trapped using 2-methyl-2-nitrosopropane (MNP) to provide the first proof that the well-known dechlorination does in fact proceed by homolytic cleavage of the carbon-chlorine bond. Continuing studies will focus on using spin traps to detect any other radical species, including active oxygen, which may be formed during photolysis of this and other phenothiazine derivatives.

Furosemide: Furosemide (4-chloro-N-furfuryl-5-sulfamolyanthranilic acid) is a diuretic which is known to cause cutaneous photosensitization. In ethanol solution, furosemide produced superoxide in the presence of oxygen and abstracted an

electron or hydrogen atom from ethanol to form the hydroxyethyl radical in deaerated solution. In aqueous solution, under anerobic conditions, a carbon-centered radical was trapped with MNP which is most likely derived from the dechlorination product. Unfortunately, due to steric hindrance, the N-O bond of the spin trap containing the unpaired electron does not lie in the plane of the aromatic ring of the furosemide; therefore very little information can be obtained from hyperfine coupling constants of the spin adduct. However, it is clear that the sulfamoylanthranilic acid portion of the molecule is mainly responsible for its radical production during photolysis. No further work is planned with is compound.

Anthracene: Anthracene, a component of coal tar, is a potent photosensitizing agent both in vivo and in vitro. Studies in mice have shown that anthracene is photocarcinogenic. Irradiation of anthracene in ethanol produced singlet oxygen as detected by the hindered amine technique. The rate of singlet oxygen production decreased in the presence of sodium azide and was enhanced when deuteroethanol was employed. Other studies have suggested that superoxide is also formed.

Anthracene caused a concentration dependent photohemolysis of human erythrocytes that was markedly enhanced by the presence of oxygen. Good protection against anthracene-induced photohemolysis was afforded by low concentrations of β-carotene, a known singlet oxygen quencher. Irradiation of human erythrocyte ghosts in the presence of anthracene resulted in lipid peroxidation, membrane protein crosslinking and a partial loss in protein secondary structure. These findings are consistent with the generation of singlet oxygen by irradiated anthracene, which in turn reacts with membrane lipids to produce peroxides that are responsible for membrane damage and ultimately hemolysis.

Instrument Development: Many environmental agents, eg. polycyclic aromatic hydrocarbons, have interesting fluorescence properties which can be used to monitor their interaction with biological molecules. Work has therefore continued to improve the utility of available fluorometers. A simple digital circuit has been designed, constructed and tested for use with our phase modulation fluorometer. This modification has made it possible to distinguish the intensity contributions form two fluorescent species provided that their excited state lifetimes differ sufficiently. A simple procedure has been devised to adjust phase modulation spectrometers so that reproducible lifetime measurements can be determined. Studies with reference compounds for lifetime studies have continued. Initial results have shown that the greatest accuracy in lifetime determinations can be achieved using reference compounds with lifetimes longer than the sample lifetimes.

B. Plans for Subsequent Year

Photolysis of skin photosensitizers

Studies will continue on the photolysis of various skin photosensitizing agents including the salicylanilides, tetracyclines, nalidixic acid, bithionol, fentichlor and N-butyl-4-chlorosalicylamide. Additional work is also planned on a series of polycyclic aromatic hydrocarbons eg. anthracene, fluoranthene, pyrene, phenanthene, chrysene, carbazole acridine and phenanthridine all of which are known components of coal tar.

A more detailed examination of anthracene will be made to identify the active oxygen species that are involved in its phototoxicity. Anthracene will be irradiated in the presence of unsaturated fatty acids and the radical species that are generated will be identified. An attempt will be made to obtain evidence for the generation of singlet oxygen by anthracene irradiation in erythrocyte ghosts and liposomal systems by detecting the presence of cholesterol hydroperoxide, a known product formed by the reaction of singlet oxygen with cholesterol. Once these techniques have been established they will be applied to other photosensitizers.

Instrumental Development

Work is planned in the development of three spectroscopic procedures to aid in the photolysis studies.

Singlet oxygen detector: Although it has been suggested that singlet oxygen may play an important role in many pathological biochemical reactions, but direct spectroscopic evidence for its existence has been lacking until recently. However, direct observation of the near-infrared (1270 nm) luminescence of singlet oxygen has made it possible to study its chemistry without chemical perturbation of a biological system.

A highly sensitive singlet oxygen detector utilizing a liquid nitrogen cooled germanium detector has therefore been designed and is being constructed. Once the responsivity of the instrument has been documented, studies of simple model homogeneous and heterogeneous (liposomal) systems will be carried out to determine the efficiency of production of singlet oxygen by photosensitizers like anthracene and fluoranthene. In addition the effect of biological quenching agents, eg. β-carotene and ascorbic acid, on the quantum yield of singlet oxygen phosphorescence will be studied.

After model studies are completed more complex systems including erythrocyte ghost membranes and isolated cells will be examined. Singlet oxygen has been implicated in the mechanism of a number of enzymes including xanthine oxidase, microsomal lipid oxidase, prostaglandin synthase, and horseradish peroxidase. Much of the evidence for singlet oxygen involvement has been based on the emission of chemiluminescence or the effects of inhibitors. With the availability of a system that permits the direct detection of singlet oxygen, the possible role of this active oxygen species in oxidative biological processes can be determined unequivocally.

Photoacoustic spectroscopy: Photoacoustic spectroscopy is a technique whereby the optical absorption spectra of opaque samples, such as solids, surfaces, gels and intact biological samples, may be obtained. In addition this technique can provide information on thermal diffusivity, depth profile of absorbing species, calorimetric properties, excited state lifetime, emission quantum yields, and the action spectrum of photoreactions. Photoacoustic spectroscopy should be ideal for studying the interaction of photosensitizing agents with the skin. A photoacoustic spectrometer has been modified for such studies and interfaced to an Apple IIe computer. Initial work has indicated that the spectra of biological samples such as bovine serum albumin can be recorded with this system. Other work with colored dyes adsorbed onto cellophane has shown that it is

possible to record absorption spectra at different levels in a multilayered system. This procedure will now be employed to study the fate of photosensitizing chemicals on skin taken from "hairless" mice. If this is successful an attempt will be made to design a cell that can be used in in vivo studies. It should also be possible, using this technique, to monitor the diffusion rate of chemicals and drugs across the skin.

Flash photolysis: Flash photolysis is a technique for the generation and detection of chemical transients. Typically there are two instrumental configurations used in flash photolysis studies, kinetic flash photolysis and spectroscopic flash photolysis. Kinetic flash photolysis involves monitoring changes in transient absorption with respect to time at a selected wavelength. As the name implies this technique can be used for decay kinetic studies. Spectroscopic flash photolysis involves the generation of a complete absorption spectrum of the transient of interest. This configuration can be a very powerful procedure for the characterization of excited states in the gas or solution phase.

The flash photolysis approach is particularly useful for examining decay from the triplet state, which commonly occurs in the microsecond region. Furthermore radicals, which often have decay times on the order of milliseconds or seconds, can also be detected by flash photolysis. We have been unable to detect the primary radicals formed during the photolysis of a number of skin photosensitizing chemicals using either conventional ESR or spin trapping. It seems reasonable to assume that such radicals, if generated, have lifetimes that are too short. The flash photolysis technique offers an additional approach to the detection and identification of such transient species. A flash photolysis unit will therefore be constructed or purchased for such studies.

C. Publications of past 18 months

Reszka, K., and Chignell, C.F.: Spectroscopic studies of cutaneous photosensitizing agents IV. The photolysis of benoxaprofen, an anti-inflammatory drug with phototoxic properities. Photochem. Photobiol., 38: 281-291, 1983.

Sik, R.H., Paschall, C.S., and Chignell, C.F.: The phototoxic effect of benoxaprofen and its analogs on human erythrocytes and rat peritoneal mast cells. Photochem. Photobiol. 38: 411-415, 1983.

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Reszka, K., Hall, R.D. and Chignell, C.F.: Quenching of the excited states of 2-phenylbenzoxazole by azide anion. A fluorescence and electron spin resonance study. Photochem. Photobiol. in press.

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Jameson, D.M., Gratton, E. and Hall, R.D.: The measurement and analysis of heterogeneous emissions by multifrequency phase and modulation fluorometry. Appl. Spectrosc. Rev. 20: 55-106, 1984.

Reszka, K., Hall, R.D. and Chignell, C.F.: Spectroscopic studies of cutaneous photosensitizing agents VII. Photoinduced reaction of benoxaprofen and 2-phenylbenzoxazole with azide anion in aerated ethanolic solutions. Photochem. Photobiol. in submission.

Paschall, C.S., Ortner, M.J. and Chignell, C.F.: The phototoxic effect of anthracene on human erythrocytes and rat peritoneal mast cells. Photochem. Photobiol. in submission.

Motten, A.G. and Chignell, C.F.: Electron spin resonance of cation radical of phenothiazine derivatives. Org. Magnet. Resonance, in submission.

Chignell, C.F.: Use of spin labels as enzyme probes. In Holtzman, J.L. (Ed.): Spin Labeling in Pharmacology. New York, Academic Press, 1984, in press.

Chignell, C.F.: Structure activity relationships in the free radical metabolism of xenobiotics. Environ. Health Perspect., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50077-02 LMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Free Radical Intermediates of Antiparasitic Drugs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert Docampo	Guest Worker	LMB	NIEHS
OTHER:	Silvia N.J. Moreno	Visiting Fellow	LMB	NIEHS
	Ronald P. Mason	Research Chemist	LMB	NIEHS

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SECTION
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INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Parasitic diseases are the most widespread of all the major human diseases. Effective treatment is nonexistent for many of them, and most of the available drugs have been shown to have mutagenic, cytotoxic, and carcinogenic activities. Recently it has become increasingly apparent that many reactive intermediates of xenobiotics are free radicals. The formation of free radicals, including oxygen-derived radicals, may lead to extensive cellular damage. The consequences of radical-initiated reactions may be the immediate death of the cells or may be more subtle and delayed as evidenced by the development of neoplasms. In a study of Trypanosoma cruzi (the agent of Chagas' disease), crystal violet, a triarylmethane dye widely used by blood banks to eliminate transmission of Chagas' disease by transfusion, was found to undergo a one-electron reduction to produce a carbon-centered free radical. Both radical formation and the trypanocidal action of crystal violet, were enhanced by light. The photodynamic action of rose bengal on T. cruzi has also been established. These observations may have important chemoprophylactic implications, since illumination of blood to be transfused may result in a better therapeutic ratio. The free radical intermediates ($O_2^{\cdot-}$, $OH^{\cdot}$) generated by human polymorphonuclear leukocytes in the presence of antibody-coated T. cruzi were identified by ESR spectroscopy. These radicals may play an important role in host resistance and/or pathogenesis of Chagas' disease. The ability of Tritrichomonas foetus (the agent of trichomoniasis in cattle) hydrogenosomal and cytosolic fractions to generate metronidazole and nitrofuran anion radicals was established. These results support the role of air oxidation as a detoxification reaction for the metronidazole anion radical and the involvement of a ferredoxin in its formation. On the other hand, redox cycling of nitrofurans, with formation of high steady state concentrations of oxygen-derived radicals, might be of toxicological significance.

A. Free Radical Intermediates of Antiparasitic Drugs

Nature of Problem: Parasitic diseases are the most widespread of all the major human diseases currently affecting about three billion people. Effective drug treatment is nonexistent for many of them, and most of the available drugs, when adequately tested, have been shown to have mutagenic, cytotoxic, and carcinogenic activities. In recent years it becomes increasingly apparent that many reactive intermediates of xenobiotics are free radicals. The formation of free radicals, including oxygen-derived radicals, may lead to extensive cellular damage. The consequences of radical-initiated reactions may be the immediate death of the cells or may be more subtle and delayed as evidenced by the development of neoplasms.

Objectives: The aim of this investigation is (a) to identify free radical intermediates generated by antiparasitic drugs and phagocytic cells in the presence of parasites by electron spin resonance spectroscopy, and (b) to investigate the ability of parasite and mammalian cells and their subcellular fractions to generate free radicals from antiparasitic compounds.

Experimental Approach and Scientific Justification: The project will be accomplished by (a) Isolation of parasite and mammalian cells and their fractions using known methods improved when necessary. (b) Determination of free radical intermediates from antiparasitic compounds by direct ESR spectroscopy or by using spin-trapping agents. (c) Study of the enzymes and cofactors involved. (d) Computer simulation of the spectra obtained to characterize radicals.

The information obtained in these studies will help make future drug development possible on a more rational basis than has been possible hitherto. In addition, the study of the mode of action of existing chemotherapeutic and chemoprophylactic agents is necessary to maximize efficacy and to minimize toxicity.

Recent Accomplishments and Signficiance

1. Light-enhanced free radical formation and trypanocidal action of gentian violet (crystal violet). In a study of intact trypanosomes, gentian violet was found to undergo a one-electron reduction to produce a carbon-centered free radical as demonstrated by electron spin resonance spectroscopy. Either reduced nicotinamide adenine dinucleotide or the reduced dinucleotide phosphate could serve as a source of reducing equivalents for the production of this free radical by homogenates of Trypanosoma cruzi. The formation of this free radical, and the trypanocidal action of gentian violet, were enhanced by light. The enhanced free radical formation may be the basic cause of the selective toxicity of gentian violet to Trypanosoma cruzi.

2. Oxygen-derived radicals from Trypanosoma cruzi-stimulated human neutrophils. We have provided biochemical and electron spin resonance spectroscopic evidence that contact of human polymorphonuclear leukocytes with anti-body coated Trypanosoma cruzi triggers the respiratory burst. Oxygen consumption, superoxide anion and hydrogen peroxide release were stimulated under conditions of polymorphonuclear leukocyte-mediated killing. This stimulation did not occur under

nonkilling conditions when antibody was omitted. A common mechanism of cytotoxicity of human polymorphonuclear leukocytes against different T. cruzi forms is suggested by the triggering of the respiratory burst by antibody-coated epimastigotes and trypomastigotes.

3. Distinct reduction of nitrofurans and metronidazole to free radical metabolites by Tritrichomonas foetus hydrogenosomal and cytosolic enzymes. Anaerobic Tritrichomonas foetus hydrogenosomes supplemented with pyruvate and CoA effectively reduce nitrofurans and metronidazole to their respective anion free radicals. Addition of purified ferredoxins from Clostridium pasteurianum for Spinacia oleracea to these preparations causes a great stimulation of metronidazole reduction, but does not affect nitrofuran reduction. A similar stimulatory effect of ferredoxin on metronidazole reduction, but not on nitrofuran reduction is observed in incubations containing purified NADPH:ferredoxin oxidoreductase from S. oleracea. NADH is less effective than pyruvate as a reducing cofactor for metronidazole and nitrofuran reduction by hydrogenosomes, and these activiti are not modified by the addition of ferredoxins. In contrast to the results observed with hydrogenosomes, the T. foetus soluble fraction supplemented with NADH or NADPH is able to reduce nitrofurans, but not metronidazole. Under aerobic conditions, the anion free radical metabolites generated from metronidazole and nitrofurans are oxidized, resulting in catalytic superoxide anion formation as detected by spin-trapping experiments. Oxygen consumption and H_2O_2 formation by T. foetus hydrogenosomes and NADPH:ferredoxin oxidoreductase are also stimulated by nitrofurans and high concentrations of metronidazole. Addition of ferredoxin enhances metronidazole-stimulated, but not nitrofuran-stimulated, oxygen consumption and H_2O_2 formation in both systems. These results support the role of air oxidation as a detoxification reaction of the metronidazole anion radical and the involvement of ferredoxin in its formation. On the other hand, redox cycling of nitrofurans with formation of high steady state concentrations of oxygen-derived radicals might be toxicological significance.

4. The photodynamic action of rose bengal on Trypanosoma cruzi. In the presence of light and oxygen, rose bengal causes oxidation damage to Trypanosoma cruzi. The production of lipid hydroperoxides was demonstrated by thin-layer chromatography, and severe ultrastructural alterations compatible with an increased permeability of the cells, which led to gradual osmotic swelling and ultimately to lysis, were observed by electron microscopy. As a result of this treatment, the infectivity of T. cruzi trypomastigotes in mice was abolished. In addition, under anaerobic conditions, rose bengal was found to undergo a one-electron reduction in intact T. cruzi epimastigotes to produce a carbon-centered free radical as demonstrated by electron spin resonance spectroscopy. The formation of this radical was also enhanced by light.

5. The literature concerning free radical formation by antiparasitic drugs and phagocytic cells was reviewed.

B. Plans for subsequent year

A natural continuation of our work on crystal violet radical formation is a detailed study of the pathways involved in this process. If some cofactors

(e.g. reducing agents such as cysteine, glutathione, ascorbic acid, etc) are essential for parasite reduction of these dyes, there should be a number of quite distinct ways of enhancing their effects while avoiding the toxic side effects. In addition the ability of mammalian cells or fractions to reduce these dyes to free radical intermediates will be studied.

Further studies on the reduction of other nitroimidazoles by Tritrichomonas foetus subcellular fractions are necessary to validate the generality of the assumptions that air oxidation is a detoxification reaction of nitroimidazole anion radicals and that ferredoxin is involved in their formation. Of particular interest will be the study of the reduction of MK-436 and HOE-239, two recently discovered 5-nitroimidazoles with potent antiparasitic activity.

C. Publications of past 18 months:

Docampo, R., Moreno, S.N.J., Muniz, R.P.A., Cruz, F.S., and Mason, R.P.: Light-enhanced free radical formation and trypanocidal action of gentian violet (crystal violet). Science 220: 1292-1295, 1983.

Docampo, R., Casellas, A.M., Madeira, E.D., Cardoni, R.L., Moreno, S.N.J., and Mason, R.P.: Oxygen-derived radicals from Trypanosoma cruzi-stimulated human neutrophils. FEBS Lett. 155: 25-30, 1983.

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Distinct reduction of nitrofurans and metronidazole to free radical metabolites by Tritrichomonas foetus hydrogenosomal and cytosolic enzymes. J. Biol. Chem., in press, 1984.

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Docampo, R., and Moreno, S.N.J.: Free Radical Intermediates in the Antiparasiti Action of Drugs and Phagocytic Cells. In Pryor, W.A. (Ed.): Free Radicals in Biology, Vol. 6, New York, Academic Press, 1984, pp. 243-288.

Docampo, R., and Moreno, S.N.J.: Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells on Trypanosoma cruzi. Rev. Infect. Dis. 6: 223-238, 1984.

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Docampo, R., and Moreno, S.N.J.: Free Radical Intermediates in the Trypanocidal Action of Drugs and Phagocytic Cells. In Bors, W., Saran, M. and Tait, D. (Eds.): Oxygen Radicals in Chemistry and Biology. Berlin, in press.

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Moreno, S.N.J., Docampo, R., and Stoppani, A.O.M.: About undesirable effects of nifurtimox and benzidazole. Medicina (Buenos Aires) 43: 291-295, 1983.

Moreno, S.N.J., Mason, R.P., Muniz, R.P.A., Cruz, F.S. and Docampo, R.: Generation of free radicals from metronidazole and other nitroimidazoles by Tritrichomonas Foetus. J. Biol. Chem. 258: 4015-4054, 1983.

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Z01 ES 50078-02 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Radical Anion Metabolites

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ronald P. Mason	Research Chemist	LMB	NIEHS
OTHER:	Silvia N.J. Moreno	Visiting Fellow	LMB	NIEHS
	Roberto Docampo	Guest Worker	LMB	NIEHS

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this study is to determine the role played by free radicals in the metabolism of xenobiotics. The anaerobic incubation of almost all nitroaromatic xenobiotics, e.g., nitrobenzene, with the microsomal, mitochondrial, or cytosolic fractions of rat liver in the presence of either NADH or NADPH, leads to a multiple-line electron spin resonance spectrum characteristic of the nitro anion free radical. We have now demonstrated nitro anion radical formation by mitochondria using endogenous cofactors. Nitro drugs do not affect mitochondrial respiration, in particular the coupling to ADP. The sites of nitro reduction, as determined by inhibitors of the mitochondrial transport chain, appear to be NADH dehydrogenase and outer-membrane NAD(P)H cytochrome c reductase.

Halogen-substituted nitro compounds are radiosensitizers and are among the most toxic nitro compounds. Loss of halide by the nitroaromatic anion forms a very reactive carbon-centered free radical, as detected by spin trapping. which reactswith cellular macromolecules. The irreversible binding of these nitro compounds to DNA, protein, etc. was inhibited by spin traps.

Free radical formation by hepatic microsomal cytochrome P-450 reduction of gentian violet, SO_2 and O_2 has also been investigated.

A. Radical Anion Metabolites

Nature of Problem: Before free radical metabolism can clearly be implicated in the origin of any toxic effect, three conditions must be met. First, the free radical metabolite must be demonstrated to exist. The best method of detecting free radicals is that of electron spin resonance spectroscopy, which permits the determination of both the free radical's structure and concentration. Second, because of toxicity of free radicals is presumably the result of purely chemical reactions, a knowledge of the radical's chemistry under physiological conditions is necessary.

The third condition is that the characteristics of the toxicity must be consistent with the known enzymatic and nonenzymatic free radical reactions. In some cases the manipulation of an animal model provides evidence that a toxicity is indeed free radical-mediated. Carbon-tetrachloride, paraquat, and nitrofurantoin poisoning represents examples of free radical-mediated toxicity which have been well studied and where all three of these conditions have been fulfilled.

Objectives: The objective of this research is to first search for free radical metabolites and then, on the basis of what is known about the free radical chemistry, to investigate the biochemical and toxicological implications of these free radical reactions.

Recent Accomplishments: Nifurtimox and nitrofurantoin were reduced by intact rat liver mitochondria to nitro anion radicals whose auto-oxidation generates superoxide anion as detected by direct electron spin resonance spectroscopy and by spin trapping experiments, respectively. Although nitro-reduction occurred in the presence of respiratory substrates, such as β-hydroxybutyrate, malate-glutamate, succinate, or endogenous substrates, nitro anion radical formation was much greater on addition of exogenous reduced pyridine nucleotides. NAD(P)H generated from endogenous $NAD(P)^+$ by intramitochondrial reactions could not be used for the NAD(P)H nitroreductase reactions unless the mitochondria were solubilized by detergent. It is concluded that the nitro reductase activity of respiratory chain enzymes is far less important than that of enzyme(s) located in the outer membrane.

In the past, studies of the anion radical metabolites formed by hepatic microsomal one-electron reduction implicated one-electron donation from NADPH-cytochrome P-450 reductase. More recently, cytochrome P-450 has been found to transfer one electron to toxic chemicals such as gentian violet, sulfur dioxide, and molecular oxygen. When gentian violet is metabolized under a nitrogen atmosphere by rat hepatic microsomes supplimented with NADPH, a single-line ESR spectrum is obtained. Either CO or metyrapone inhibits radical formation by 50%, suggesting cytochrome P-450 involvement. Under an atmosphere of nitrogen, rat liver microsomal incubations containing bisulfite (aqueous sulfur dioxide) and NADPH form a free radical with a single line ESR spectrum. The results imply that cytochrome P-450 reduces bisulfite to the sulfur dioxide anion radical. Oxygen completely inhibited

the formation of this radical, which is consistent with oxygen being a competitive inhibitor for the reduced heme of cytochrome P-450. We have used the spin trapping technique to demonstrate the microsomal reduction of oxygen to superoxide, which we also found to be inhibited by CO and metyrapone.

Experimental Approach: Free radicals may be detected, identified and quantitated by ESR spectroscopy. However, since most free radicals generated photolytically are chemically reactive they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from ESR spectrum of the resultant spin adduct.

B. Plans for Subsequent Year

The previously unsuspected role of NAD(P)H-cytochrome c reductase of the mitochondrial outer member in the reduction of xenobiotics will be extended to the one-electron reduction of oxygen and anticancer quinone drugs to superoxide and semiquinones, respectively.

C. Publications of past 18 months

Peterson, F.J., Combs, G.F., Jr., Holtzman, J.L., and Mason, R.P.: Effects of selenium and vitamin E deficiency on nitrofurantoin toxicity in the chick. J. Nutrition, 112: 1741-1746, 1982.

Peterson, F.J. Combs, G.F., Jr. Holtzman, J.L., and Mason, R.P.: Metabolic activation fo oxygen by nitrofurantoin in the young chick. Toxicol. and Appl. Pharmacol. 65: 162-169, 1982.

Docampo, R., Moreno, S.N.J., and Mason, R.P.: Generation of free radical metabolites and superoxide anion by the Calcium indicators arsenazo III, artipyrylazo III, and murexide in rat liver microsomes. J. Biol. Chem. 258: 14920-14925, 1983.

Mason, R.P.: Assay of in situ radicals by electron spin resonance. In Packer, L. (Ed): Oxygen Radicals in Biological Systems, Methods in Enzymology, 105, New York Academic Press, 1984, pp. 416-422.

Kalyanaraman, B., Mottley, C. and Mason, R.P.: On the use of organic extraction in the spin-trapping technique as applied to biological systems. J. Biochem. Biophys. Meth. 9: 27-31, 1984.

Moreno, S.N.J., Mason, R.P., and Docampo, R.: Reduction of nifurtimox and nitrofurantoin to free radical metabolites by rat liver mitochrondria. Evidence of an outer membrane located nitroreductase. J. Biol. Chem. 259: 6298-6305, 1984.

Mason, R.P. and Josephy, P.D.: Free radical mechanism of nitroreductase. In Rickert, D. (Ed): Toxicity of Nitroaromatic Compounds, Hemisphere, New York (in press).

Josephy, P.D. and Mason, R.P.: Chemical and enzymatic nitroreduction; Free radical and diamagnetic products of nitroimidazoles; In Anders, M.W. (Ed): Bioactivation of Foreign Compounds, Academic Press, New York (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50079-02 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Free Radical Metabolite Formation by Peroxidases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ronald P. Mason	Research Chemist	LMB	NIEHS
OTHER:	Volker Fischer	Visiting Fellow	LMB	NIEHS
	Paul West	Guest Worker	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Molecular Biophysics

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

The objectives of this investigation are to detect quantitate and identify free radical metabolites generated from endogenous and exogenous chemical by peroxidase enzymes. Electron spin resonance (ESR) investigations of the prostaglandin hydroperoxidase and a model enzyme system, horseradish peroxidase, have demonstrated the enzymatic formation of free radical metabolites. The oxidation of benzidine and several derivatives was studied using horseradish peroxidase and prostaglandin synthase. Benzidine was metabolized to a radical cation and a charge-transfer complex composed of the benzidine and its two electron (di-imine) oxidation product. The two-electron oxidation product, the di-imine, is a resonance structure of the nitrenium ion, the proposed ultimate carcinogenic metabolite of aromatic amines. Mono-acetylbenzidine is a relatively poor peroxidase substrate. Electron spin resonance spectroscopy, employing a millisecond time scale fast-flow method, has revealed the formation of a transient phenoxy radical in the reaction of acetaminophen with horseradish peroxidase/H_2O_2 and bovine lactoperoxidase/H_2O_2. The short-lived radical is clearly distinguished from the persistent paramagnetic melanin polymers that are generated by prolonged incubation of acetaminophen in the presence of oxidizing enzymes. Ram seminal vesicles and acetaminophen under fast-flow conditions demonstrated the oxidation of acetaminophen to its phenoxyl free radical by the mammalian enzyme prostaglandin hydroperoxidase. Sulfur-centered free radicals have been detected when cysteine was incubated with horseradish peroxidase and H_2O_2. In the presence of either molecular oxygen or hydrogen peroxide the thiyl radical was converted to the cysteine sulfonic and sulfinic acids. Reduced glutathione (GSH) was also oxidized to a sulfur-centered radical (GS·) by horseradish peroxidase and H_2O_2. Since cysteine and glutathione play an important role in the structure and function of sulfhydryl-containing proteins, these oxidation reactions may modulate the biological function of these compounds.

A. Free Radical Metabolite Formation by Peroxidases

Nature of Problem: Free-radical intermediates have been found in the metabolic pathways of a wide variety of organic compounds, and, on the whole, free radical metabolites seem to be implicated in the toxic effects of those xenobiotics which are metaoblized to free radicals. In general, these toxic effects appear to be the result of the purely chemical reactions of the free radical, therefore a knowledge of the chemistry of the free radical, was well as a demonstration of its existence in the metabolic pathway, is necessary before free-radical metabolism can be implicated in the origin of a toxic effect. In a few cases animal model studies have indicated that the toxicity of a xenobiotic is indeed free-radical mediated.

Objectives: The objectives of this project are (a) to use electron spin resonance (ESR) and other techniques to detect, identify and quantitate free radical metabolites generated by the metabolism of endogenous and exogenous compounds. (b) to implicate free radical metabolites in the known toxic effects of the parent chemical and (c) to determine the biological role played by free radicals derived from endogenous compounds.

Experimental Approach: Free radicals may be detected, identified and quantitated by ESR spectroscopy. However, since most free radicals generated photolytically are chemically reactive they cannot always be observed by direct ESR. For such radicals the technique of spin trapping may be employed. This procedure uses diamagnetic organic molecules (spin traps) that react with free radicals to produce stable nitroxide radicals (spin adducts). The identity of the parent radical may often be determined from ESR spectrum of the resultant spin adduct.

Recent Accomplishments and Significance:

Acetaminophen: There is considerable evidence for the in vivo oxidation of acetaminophen to an arylating intermediate, N-acetyl-p-benzo-quinoneimine, which may bind to tissue macromolecules and cause hepatic necrosis. We have demonstrated by fast-flow electron spin resonance spectroscopy that the horseradish peroxidase/hydrogen peroxide system oxidized acetaminophen to a transient phenoxyl free radical, which is thought to be the intermediate in the oxidation of acetaminophen to N-acetyl-p-benzoquinoneimine. Both an over-modulated and high resolution spectrum have been obtained under fast-flow conditions. A more stable derivative of acetaminophen, with methyl groups placed at the ortho positions of the oxygen-centered free radical, has been prepared and used to study the dependence of free radical formation on enzyme and substrate concentration. The enzymatic oxidation of 3,5-dimethylacetaminophen with the horseradish peroxidase/hydrogen peroxide system forms a phenoxyl free radical metabolite. The structure of this free radical was established by a complete analysis of the ESR spectrum and confirmed by deuterium isotope substitution. Concomitant with phenoxyl radical formation, N-acetyl-3,5-dimethyl-p-benzoquinone imine was detected by optical spectroscopy. The phenoxy free radical was also formed by comproportionation in solutions of the quinone imine and added 3,5-dimethylacetaminophen. In contrast to acetaminophen, these imine and radical metabolites are stable and can be detected without resort to rapid-mixing techniques. The high reactivity of the free radical metabolite of acetaminophen may be related to the toxicity of this drug in vivo.

Cysteine: The sulfhydryl group of cysteine plays many important roles in both the structure and function of proteins, and the oxidation of L-cysteine often modulates these roles. The oxidation of L-cysteine is known to be responsible for the radioprotection of intracellular GSH, the bactericidal effect of cysteine, and the loss of ATP and GSH from isolated rat hepatocytes. Although the importance of free radicals in the radiolytic and metal ion oxidation of L-cysteine is clear, no direct evidence of a role for cysteine-derived free radicals in an enzymatic reaction has been reported. We have used the spin-trapping ESR technique to study free radical metabolites formed via the oxidation of cysteine by the peroxidase prototype, horseradish peroxidase. The oxidation of L-cysteine by horseradish peroxidase in the presence of oxygen forms a thiyl free radical, which reacts with spin trap 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) to form the DMPO-cysteine thiyl radical adduct. This radical adduct has a distinctive ESR spectrum (a_{β}^{N} = 15.3 and a^{H} = 17.0 G). Omission of hydrogen peroxide under anaerobic conditions results in a dramatic decrease in signal intensity, indicating that hydrogen peroxide is necessary for the adduct to form anaerobically. However, aerobic incubations without added hydrogen peroxide show signal intensities comparable to those obtained anaerobically with hydrogen peroxide. Thus, it appears that oxidation of L-cysteine by horseradish peroxidase to the thiyl free radical is dependent upon peroxide(s) that can be formed from atmospheric oxygen. With addition of catalase, 80% inhibition of adduct accumulation was obtained. Heat-denatured catalase had no effect. Studies with a Clark oxygen electrode show that oxygen is consumed in incubations of cysteine and horseradish peroxidase, presumably due to reactions of the thiyl free radical. The oxygen consumption is inhibited by DMPO as expected. Addition of molecular oxygen or hydrogen peroxide to the thiyl radical ultimately leads to cysteine sulfinic and cysteine sulfonic acids. The oxidation of cysteine sulfinic acid leads to the formation of both sulfur-centered and carbon-centered free radicals. GSH is also oxidized to GS$^{\cdot}$ by horseradish peroxidase and hydrogen peroxide, but not by gluthathione peroxidase and hydrogen peroxide. Thiyl radical formation by horseradish peroxidase is inhibited by glutathione peroxidase after a rapid stimulation phase.

Benzidine: The oxidation of benzidine and several derivatives was studied using horseradish peroxidase and prostaglandin synthase. Benzidine was metabolized to a radical cation and a charge-transfer complex composed of the benzidine and its two electron (di-imine) oxidation product. The two-electron oxidation product, the di-imine, is a resonance structure of the nitrenium ion, the proposed ultimate carcinogenic metabolite of aromatic amines. Mono-acetylbenzidine is a relatively poor peroxidase substrate.

B. Plans for Subsequent Year

This work is being extended to sulfhydryl drugs used to treat hyperthyroidism. The mechanism of action of these drugs is thought to involve thyroid peroxidase. The oxidation of GSH by prostaglandin hydroperoxidase is being considered as the endogenous reducing cofactor responsible for the conversion of PGG_2 to PGH_2.

C. Publications of past 18 months

Josephy, P.D., Mason, R.P., and Eling, T.: Chemical structure of the adducts formed by the oxidation of benzidine in the presence of phenols. Carcinogenesis 3: 1227-1230, 1982.

Mottley, C., Trice, T.B., and Mason, R.P.: Direct detection of the sulfur trioxide radical anion during the horseradish peroxidase-hydrogen peroxide oxidation of sulfite (aqueous sulfur dioxide). Mol. Pharmacol. 22: 732-737, 1982.

Kalyanaraman, B., Mottle, C., and Mason, R.P.: A direct electron spin resonance and spin-trapping investigation of peroxyl free radical formation by hematin/hydroperoxide systems. J. Biol. Chem. 258: 3855-3858, 1983.

Josephy, P.D., Eling, T.E., and Mason, R.P.: Oxidation of p-aminophenol catalyzed by horseradish peroxidase and prostaglandin synthase. Mol. Pharmacol. 23: 461-466, 1983.

Josephy, P.D., Eling, T.E., and Mason, R.P.: Co-oxidation of benzidine by prostaglandin synthase and comparison with the action of horseradish peroxidase. J. Biol. Chem. 258: 5561-5569, 1983.

Josephy, P.D., Eling, T.E., and Mason, R.P.: An electron spin resonance study of the activation of benzidine by peroxidases. Mol. Pharmacol. 23: 766-770, 1983.

Eling, T., Boyd, J., Reed, G., Mason, R.P., and Sivarajah, K.: Xenobiotic metabolism by prostaglandin endoperoxide synthetase. Drug Metabol. Rev. 14: 1023-1053, 1983.

Kalyanaraman, B., Sivarajah, K., Eling, T.E., and Mason, R.P.: A free radical mediated cooxidation of tetramethylhydrazine by prostaglandin hydroperoxidase. Carcinogenesis 4: 1341-1343, 1983.

Harman, L.S., Mottley, C. and Mason, R.P.: Free radical metabolites of L-cysteine oxidation. J. Biol. Chem. 259: 5606-5611, 1984.

West, P.R., Harman, L.S., Josephy, P.D., and Mason, R.P.: Acetaminophen: enzymatic formation of a transient phenoxyl free radical. Biochem. Pharm., in press.

Fischer, V., and Mason, R.P.: Stable free radicals and benzoquinone imine metabolites of an acetaminophen analogue. J. Biol. Chem. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50080-02 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Environmental Health Applications of Mass Spectrometry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Ronald Hass	Research Chemist	LMB
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	Carol E. Parker	Chemist	LMB
	Donald J. Harvan	Chemist	LMB

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Mass Spectrometry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

More than 2000 sample analyses were performed in support of other NIEHS scientists. Collaborative research problems have included: characterization of metabolites of phthalates and diethylstilbestrol, measurement and identification of chlorinated dibenzo-p-dioxins and dibenzofurans in soil and animal tissues; estimation of 3-methoxy-4-hydroxyphenylglycol in rat brain; identification of physalemine-like peptides and epidermal growth factor in tissues; identification of corticosterone metabolites in mammary gland; and characterization of phospholipids.

A. Environmental Health Applications of Mass Spectrometry
Nature of Problem: The problems approached in this project have as common denominators the need to know either what is in a particular sample or the concentration of selected components of a complex sample.

The objectives of this project is to provide research support in analytical chemistry to problems in environmental health sciences. The most important aspects of this objective are quality and timeliness. This includes the availability of adequate equipment and qualified mass spectrometrists to participate in collaborations.

The experimental approach involves mass spectrometry using a variety of ion sources including electron impact (EI), chemical ionization (CI) sampling positive or negative or negative ions, and fast atom bombardment using direct probe, gas chromatographic (GC), or high performance liquid chromatographic (HPLC) inlets and has been applied to a variety of environmental/biomedical problems. Routine service samples are analyzed in a timely manner for any NIEHS scientist requesting support. More extensive collaborative research projects are enthusiastically executed with Institute Staff.

The recent accomplishments and their significance are discussed below for each study. For those cases in which the collaborative was initiated by another NIEHS scientist, the project will simply be mentioned along with the name of the originating scientist so as to avoid needless duplication.

SERVICE: More than 2000 sample analyses were performed in support of other NIEHS scientists. In the majority of cases, sample turn-around was less than one working day with requests rarely pending for as long as three days. The majority of these samples were for EI analysis using GC. A substantial number of these required exact mass measurements. Fast atom bombardment is also increasing in popularity as a service function.

COLLABORATIVE RESEARCH: These research projects are, in general, originated by scientist not in the Mass Spectrometry Group. For this reason, this report will be restricted to only the analytical chemistry aspects of the findings with a reference to the originating party.

METABOLITE IDENTIFICATION:

1. Polar metabolites of DEHP (P.W. Albro)

2. Metabolites of Di-(5-Hexenyl) Phthalate (P.W. Albro)

3. Metabolites of Di-(9-Decenyl) Phthalate (P.W. Albro)

4. Metabolites of 4,4'-Thiobis-(6-Butyl-m-Cresol) (H.B. Matthews)

5. Metabolites of DES (J. McLachlan)

QUANTITATIVE ANALYSIS:

1. TCDDs in soil; bioavailability of TCDD (E.E. McConnell)

2. TCDDs in biological samples; Induction of enzymes by TCDD bound to soil (G.W. Lucier, E.E. McConnell).

3. MHPG in rat brain; distributation of neurometabolites (J. Hong, P. Chem.).

CHARACTERIZATION OF BIOMOLECULES:

1. Epithelial growth factor (EGF) (O. Hernandez, R. DiAugustine)

2. PLIM (a peptide) (W. Wilson)

3. Transformation products of corticosterone in mammary glands (W. Pearlman, Department of Pharmacology, University of North Carolina at Chapel Hill). It has been found that a corticobinding protein resembling, if not identical to, serum corticosteroid-binding globulin appears in the milk of lactating rats. This suggests that corticosteroid-binding globulin arising from systemic circulation enters the lumen of the alveolus via an intracelluar route and thus is implicated in the transport in the corticosterone into the alveolar cells. The binding was found to involve the formation of 21-acyl derivatives of the corticosterone. The major acyl product was found to be oleate.

4. Characterization of phospholipids. (Professor C. Djerassi, Department of Chemistry, Stanford University). Phospholipids, along with steroids, are among the main classes of lipids in the cell membranes of living organisms. The characterization of these compounds is important for the understanding of a variety of problems involving transport through cell membranes. In this study, it was found that fast atom bombardment and direct chemical ionization mass spectrometry give complimentary information. Should one be restricted to one technique, the FAB was the more informative.

B. Plans for Subsequent Year:
The essential features of the research support program will be continued along present lines. The increasing demand for mass spectrometry in collaborative research is causing some delays in the more routine service applications due to instrument availability. This problem is being addressed by the addition of a very simple GC/MS system so that the load for less demanding work will be removed from the more sophisticated mass spectrometers. An experiment has been conducted over the past two years to address the problem of manpower to perform the measurements. This is to assist biomedical groups who anticipate a large useage of mass spectrometry in their research to recruit a post-doctoral mass spectrometrist (either as a Visiting Fellow or Special Expert) to work on their problem in collaboration with the MSG. This has been very successful and will be expanded as demand justifies it.

C. Publications of past 18 months:

Smith, R.W., Parker, C.E., Matthews, H.B. and Hass, J.R.: Identification of the major metabolite of 4,4'-thiobis-(6-t-butyl-m-cresol). Biomed. Mass Spectrom. (submitted).

Ayanoglu, E., Wegmann, A., Pile, O., Marbury, G.D., Hass, J.R. and Djerassi, C.: Mass spectrometyr of phospholipids. Some applications of desorption chemical ionization and fast atom bombardment. J. Am. Chem. Soc. (in press)

Rumbaugh, R.C., McConnell, E.E., McCoy, Z., Harvan, D.J., Hass, J.R., Albro, P.W. and Lucier, G.W.: Induction of heptaic aryl hydrocarbon hydroxylase in Sprage Dawley rated by administration of soil contaminated with 2,3,7,9-tetrachlorodibenzo-p-dioxin (TCDD). Science (in press)

Walther, H.J., Parker, C.E., Harvan, D.J., Voyksner, R.D., Hernandez, O., Hagler, W.M., Hamilton, P.B. and Hass, J.R.: Analysis of aflatoxins and reaction products of sodium bisulfite addition with aflatoxins by fast atom bombardment mass spectrometry. J. Ag. Food Chem. 31: 169-171, 1983.

McConnell, E.E., Lucier, G.W., Rumbaugh, R.C., Albro, P.W., Harvan, D.J., Hass, J.R. and Harris, M.W.: Dioxin in soil: Bioavailability after ingestion by rats and guinea pigs. Science 223: 1077-1079, 1984.

Albro, P.W., Corbett, J.T., Marbury, G.D., and Parker, C.E.: Metabolism of Di-(5-hexenyl) phthalate and di-(9-decenyl) phthalate in the rat. Xenobiotica (in press).

Albro, P.W., Parker, C.E., Marbury, G.D., Hernandez, O., and Corbin, F.T.: Spectrometric characterization of metribuzin and its metabolites. Appl. Spectrosc. (in press).

Parker, C.E., Albro, P.W., Bobenreith, M.J., Cochran, T.W., and Robinson, J.D.: Quantitation of halogenated compounds by GC/MS. J. Chromatog. 278: 1-11, 1983.

Parker, C.E., Degen, G.H., Abustrit, E.O., and Corbin, F.T.: The determination of metribuzin and its metabolites by high pressure liquid chromatography. J. Liquid Chromatography 6: 725-742, 1983.

Tondeur, Y., Hass, J.R., Harvan, D.J., McKinney, J.D. and Albro, P.W.: Determination of suspected toxic impurities in firemaster FF-1 or BP-6 by capillary column gas chromatography/high resolution mass spectrometry. J. Ag. Food Chem. (in press).

Albro, P.W., Tondeur, Y., Marbury, G.D., Jordan, S., Schroeder, J., and Corbett, J.: Polar metabolites of DEHP in the rat. Biophy. Biochem. Acta. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50081-02 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Improvements in Fluorometric Instrumentation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert D. Hall	Staff Fellow	LMB	NIEHS
OTHER:	Colin F. Chignell	Chief, LMB	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Molecular Biophysics

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been combined with Z01 ES 50046-06 LMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 50082-01 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Tumor Promoters and Antipromoters

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Phillip W. Albro	Research Chemist	LMB	NIEHS
Other:	Ronald P. Mason	Research Chemist	LMB	NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Biophysics

SECTION

Bio-organic Chemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tumor promoters can often be differentiated by their effects in two model systems: polynuclear aromatic hydrocarbon (PAH)-initiated carcinogenesis in mouse skin and nitrosamine-initiated carcinogenesis in regenerating rat or mouse liver. Phorbol esters promote in both systems, some phthalate esters promote the latter and fail to affect the former, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) promotes the latter and is an antipromoter for the former, while polychlorinated biphenyls (PCBs) may promote or act as an antipromoter for both depending on the dosages tested. A comparison of these classes of environmental pollutants may provide mechanistic insights absent when any one is studied by itself. This is a new project that is still largely in the planning and preparation stages.

A. Studies on Tumor Promoters and Antipromoters

Nature of Problem: Several classes of common environmental pollutants are positive in model system tests for tumor promotion but negative in tests for initiation, genotoxicity, and mutagenesis. Some of these classes of compounds, for example chlorinated dibenzo-p-dioxin, polychlorinated biphenyls, and phthalate diesters, also act as antipromoters in other protocols, antagonizing the promotion by classical promoters such as the phorbol esters. This dichotomy makes it conceptually difficult to visualize the mechanism(s) of action of a member of these classes.

Objectives: (1) Considering the chemical classes polychlorinated dibenzo-p-dioxins (esp. 2,3,7,8-TCDD), polychlorinated biphenyls, phorbol esters and phthalate diesters (esp. DEHP), to identify biochemical systems with which more than one class interacts and which may have some relationship to promotion or antipromotion; (2) to form and test hypotheses concerning the biochemical or molecular level mechanisms by which these chemcials exert their promoter or antipromoter activity. More specific objectives will be defined as the project develops.

Expermimental Approach: Since there are already a number of systems with which phorbol ester promoters are known to interact, including specific receptors, lipid peroxidation in target tissues, specific hydrolase enzymes related to species differences in susceptability, effects on T-killer cell production (immune surveillance), and several others, the other classes of test compounds will be tested for ability to interact with these model systems. In most cases radioisotopically labeled forms of the test compounds are used to measure binding, metabolic alteration, etc. Possible effects of the test compounds on metabolic activation of initiator compounds are investigated using radiolabeled initiators in tests of covalent binding to macromolecules. Lipid peroxidation is studied by ESR spectrometry, measurement of aldehyde production, conjugation of double bonds, disappearance of polyunsaturated fatty acids, and production of fluorescent pigments.

Recent Accomplishments and Significance: Experimental results are limited, since the project is just beginning. 2,3,7,8-TCDD failed to affect production of detectable free radicals in a peroxidase-supported cooxidation system. Although there is evidence for TCDD-stimulated lipid peroxidation *in vivo* (production of lipofuscin pigments at a greatly accelerated rate) in rats, TCDD failed to stimulate lipid peroxidation in an NADPH-dependent, microsome-based model system *in vitro*. Phorbol diester hydrolase activity does not co-distribute with phthalate diester hydrolase in rat and mouse skin.

The PCBs and phthalate esters are the two most widespread of all environmental pollutants, and both are commonly found in human tissues. TCDD is the most acutely toxic of man-made chemicals in the environment (LD_{50} 0.5 μg/Kg in guinea pigs) and one of the most potent known promoters of hepatocarcinogenesis. The fact that these chemcials may act as promoters or antipromoters depending on the nature of the initiator (e.g. TCDD can block the promoter action of phorbol esters in mouse skin) makes predictions of the probable consequences of exposure to these compounds nearly impossible. This situation will not be alleviated

until the mechanisms underling these biological activities are understood.

B. Plans for Subsequent Year

1. Since both 2,3,7,8-TCDD and di-(2-ethylhexyl) phthalate (DEHP) are considered tumor promoters of hepato-carcinogenesis following initiation by diethylnitrosamine (DEN) (Pitot et al., Cancer Res. 40: 3616, 1980; Ward et al., Carcinogenesis 4: 1021, 1983) but the polychlorinated biphenyl product Kanechlor 500 acts as an antipromotor in this system (Makiura et al., JNCI 53: 1253, 1974), these three environmental pollutants will be compared in all promoter studies. TCDD acts as an antipromoter when benzo(a)pyrene or 7,12-dimethylbenz(a)anthracene are used as innitiators (Cohen et al., Cancer Res. 39: 4027, 1979). Antipromotion by DEHP has been claimed by DeAngelo and Garrett (Cancer Lett., 20: 199, 1983). TCDD is not significantly metabolized by rodents (except hamsters), DEHP is rapidly metabolized, and PCBs are slowly metabolized. Comparisons of these three should provide insights lacking in studies of either one alone.

One possible mechanism for hepatocarcinogenesis by diethylnitrosamine is as follows:

1. $(C_2H_5)_2NNO \rightarrow \rightarrow [C_2H_5]^+$
2. $[C_2H_5]^+$ + Enzymes → inactive enzymes
3. $[C_2H_5]^+$ + DNA → "Altered" DNA
4. "Altered" DNA $\xrightarrow{\text{enzyme}}$ "repaired" DNA
5. "Altered" DNA $\xrightarrow[\text{division}]{\text{cell}}$ "mutated" DNA $\xrightarrow[\text{division}]{\text{more cell}}$ cancer.
6. Cancer cells $\xrightarrow[\text{surveillance"}]{\text{"immune}}$ destroyed cancer cells.

TCDD, through its induction of liver enzymes, might stimulate step 1. This would increase step 3 (more altered DNA) and step 2 (less repair enzymes), leading to less repair (step 4). There is no evidence for a significant simulation of step 5 by TCDD, as there is for PCBs and phorbol esters, nor does DEHP stimulate cell division even in partially hepatectomized rats. However, there is a major toxic effect of TCDD on the thymus in most species, so TCDD (and PCBs but not DEHP which has no reported effect on the immune system) might be expected to interfere with step 6 above also.

In future studies we plan to examine the effects of the test compounds on the biochemical reactions of DEN in rodent liver, using [^{14}C-Ethyl]-DEN. Association of covalently bound ^{14}C with liver proteins and DNA will be followed as a function of time. Incubation of pre-alkylated DNA with liver preparations from animals pretreated with the test compounds will be followed to detect effects on excision repair. An attempt will be made to establish a collaborative relationship to examine the effects of these test compounds on the generation of T-killer cells against syngeneic tumor cells in tissue culture. This test has been successful in visualizing a detremental effect of phorbol ester promoters on immune surveillance (step 6 above) (Frederickson and Bennett, Cancer Res. 42: 3601, 1982).

2. Some tumor promoters, again notably the phorbol esters, are associated with lipid peroxidation in the target organs (e.g. Logan; et al., Cancinogenesis 1: 1303, 1982). TCDD also appears to have some association with lipid peroxidation

events in vivo though this has not been demonstrated in vitro. We plan to evaluate the effect of TCDD on the non-heme-iron-dependent lipid peroxidation system involving hepatic microsomes in vitro, since it has been claimed (Sweeney et al., Science 204: 332, 1979) that iron deficiency reduces the biological activity of TCDD in vivo.

None of these studies involve an assumption that TCDD acts by a mechanism corresponding to that of the phorbol esters (although DEHP might possible do so). However, the phorbol esters have been helpful in identifying sensitive and responsive biological systems, which should be examined before totally new systems are sought.

Publications of past 18 months

Albro, P.W., Corbett, J.T., Schroeder, J.L., and Jordan, S.T.: Incorporation of radioactivity from labeled di-(2-ethyl-hexyl) phthalate (DEHP) into DNA of rat liver in vivo. Chem.-Biol. Interact. 44: 1-16, 1983.

Serabjit-Singh, C.J., Albro, P.W., Robertson, I.G.C., and Philpot, R.M.: Interactions between xenobiotics that increase or decrease the levels of cytochrome P-450 isozymes in rabbit lung and liver. J. Biol. Chem. 258: 12827-12834, 1983.

Chae, K., Albro, P.W., Luster, M.I., and McKinney, J.D.: Screening assay for the tetrachlorodibenzo-p-dioxin receptor using the 125-I-Iodovaleramide derivative of trichlorodibenzo-p-dioxin as the binding ligand. Int. J. Environ. Anal. Chem. (in press).

Bend, J.R., Foureman, G.L., Ben-Zvi, Z., and Albro, P.W.: Heterogeneity of hepatic aryl hydrocarbon hydroxylase activity in feral winter flounder: Relevance to carcinogenesis. Natl. Cancer Inst. Monographs 65: 359-370, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80008-10 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis of Prostaglandins, Hydroxy-Fatty Acids and Leukotrienes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Prostaglandin Biochemistry

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	3.7	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pulmonary tissue produces high amounts of prostaglandins (PG), leukotrienes (LT), and hydroxy fatty acids (HFA) in response to a number of stimuli or pathological states. These lipids have diverse biological activities. The goal of this study is two-fold: first to develop an understanding of factors that control the biosynthesis of these lipids and second to determine their role in the secretory and inflammatory processes of the lung.

We have chosen to study the biosynthesis of these lipids in dog tracheal epithelial cells and to determine their role in control of Cl^- and mucus secretion. Dog trachea cells make primarily PGD_2 and a number of LTs. LTC_4, LTB_4 and two unknown LTs are also produced. While Cl^- secretion appears to be primarily under control of PGD_2 formation, other data suggest a regulatory role for the LTs. We intend to characterize further the LTs formed and relate them to Cl^- secretion.

We are also examining the formation of LTs in response to asbestos exposure. Cultures of rat pulmonary macrophages release LTs in response to asbestos exposure. Macrophages release primarily LTB_4 but some LTC_4 is also liberated. We intend to characterize further the products released by asbestos from rat macrophages and to explore the possible relationship between macrophages and the development of asbestosis.

We have also found that mouse skin is very rich in prostaglandin synthase and essentially devoid of lipoxygenase activity. The major cyclo-oxygenase product is PGE_2 as characterized by high pressure liquid chromatography and gas chromatography-mass spectrometry.

A. Biosynthesis of Prostaglandins, Hydroxy-Fatty Acids and Leukotrienes

Nature of Problem: Arachidonic acid is metabolized to prostaglandins, thromboxanes, hydroperoxy and hydroxy fatty acids and leukotrienes. These lipid have an extremely diverse biological activities and have been implicated in numerous biological processes. Little is known about the biochemistry of arachidonic acid metabolism and even less is known about mechanisms that control arachidonic acid metabolism. Our aim is to develop an understanding of these processes particularly as related to environmental-induced toxicity or disease states. Cystic fibrosis is a pulmonary disease associated with disturbance in water and electrolyte movement and increased mucus secretions. These processes appear to be controlled or modulated by arachidonic acid metabolites. Dog tracheal epithelials cell are model for this disease state. Also the presence of peroxides in tissues alters arachidonic acid metabolism particularly the formation of PGI_2. The hydroperoxidase of prostaglandin synthase, which reduces peroxides may be a control point for arachidonic acid metabolism.

Objectives: The objectives of this study are: (a) to examine the factors that control PGI_2 biosynthesis, particularly the role of peroxides in this process (b) to characterize the arachidonic acid metabolites by dog tracheal cells and study the relationship to Cl ion and mucus secretion (c) to characterize the arachidonic acid metabolites in isolated cells of potential usefullness in studies of chemical co-oxidation and (d) to o study mechanisms involved in prostaglandin biosynthesis

Experimental Approach: Prostaglandin (PG), thromboxane (TX), hydroxy-fatty acid (HFA) and leukotriene (LT) synthetase activities were measured in vitro using the microsomal protein from a variety of tissues and organs as an enzyme source. ^{14}C-Arachidonic acid (AA) or prostaglandin endoperoxides were incubated at 37°C for various times and under several conditions. After incubation, the PGs and LTS were removed by solvent extraction, separated by high pressure liquid chromatography, and estimated by liquid scintillation techniques. Metabolites were also separated and quantitated by a GC method recently developed. Metabolites were characterized by GC-MS techniques.

Biosynthesis was studied by adding labeled arachidonic acid to cells isolated from rat, dog and rabbit lung. PGs were separated by HPLC. In some experiments cells were maintained in culture for various lengths of time, trypsinized, counted, sonicated, and PGs analyzed. PG biosynthesis was also studied using microsomal fractions. Alveolar macrophages were isolated from rat lung and cultured using standard procedures. Intact dog trachea epithelial cells were isolated and Cl^- secretion measured by electrical potential. Mouse skin cells were isolated and separated into purified cell fractions.

Recent Accomplishments: An HPLC method was developed which permits rapid and nearly complete separation of major arachidonic acid metabolites. The method is based on the use of radiolabeled arachidonic acid as a means of quantitating the metabolites. Leukotrienes are also measured by UV absorbances at 280 nm.

Recently we have developed a GC method for the separation of PGs, LT's and hydroxy fatty acids. These lipids are converted to pentafluorobenzyl oxime derivatives which can be gas chromatographed. The pentafluro group allow quantitation by electron capture technique. These derivatives can be used for mass spectrometry analysis of the metabolite. This method allows us to separate, quantitate and identify metabolites without the use of radioisotopes.

Mouse skin epidermal cells convert arachidonic acid to PGE_2 and a number of lesser unidentified metabolites. PGE_2 was characterized by HPLC and GG-MS. These cells have very high PGE_2 biosynthetic activity and appear to be very suitable for studying the co-oxidation of chemicals.

We have characterized arachidonic acid metabolism by dog tracheal cells. These cells make PGD_2, LTC_4 and LTB_4 plus two unidentified metabolites. The time course for the formation of PGD_2 correlates with chloride secretion. Indomethacin inhibits PGD_2 formation and Cl^- secretion. PGD_2 added to cells increases chloride secretion. Basal level of PGD_2 formation also correlates with basal Cl secretion. Arachidonic acid metabolites were characterized by HPLC, enzymatic metabolites, UV spectrometry and GC-MS.

Rat alveolar macrophages make LTC_4, LTB_4 and TXA_2 in response to a calcium ionophore. Exposure to asbestos produced a rapid release of AA and formation of LTB_4 and TXA_2. Cyclo-oxygenase products were more predominant. Exposure to iron particles also releases AA and results in the formation of TXA_2 and LTB_4. The response to iron is greater than the response to asbestos due to cytotoxicity of the asbestos. The response to asbestos does not appear to be related to phagocytosis but the binding of the asbestos to cell membranes.

We have also studied the effect of vitamin K on PGI_2 biosynthesis. The addition of vitamin K_1 to endothelial cells, produced a dose dependent inhibition of PGI_2 formation. Moreover, the response to K_1 was biphasic in that a time dependent stimulation was observed at some doses of K_1. Inhibition of biosynthesis could not be demonstrated by in vitro subcellular preparations. We have also examined the effect of a phenylbutazone-dependent peroxyl radical on prostaglandin and prostacycline synthesis. PGI_2 biosynthesis was inhibited at low peroxyl radical concentrations, but as the radical concentration was increased PGS is also inhibited. This shows the importance of peroxide in modulating arachidonic acid metabolism.

B. Plan for Subsequent Years

To study arachidonic acid in human bronchial epithelial cell and relate to Cl and mucus secretion. We intend also to obtain tissues from cystic fibrosis patients and examine arachidonic acid metabolism. This may give a clue to distubance in the metabolism responsible in part for the disease.

To characterize the unknown arachidonic acid metabolite by dog tracheal epithelial cells and to test for biological activity on Cl ion and mucus secretion.

To fully characterize the role of vitamin K in PGI biosynthesis and to elucidate mechanism responsible.

To study the effect of phenylbutazone peroxyl radical on arachidonic acid metabolism using endothelial cell as a model system. We also will study the effect of the radical on leukotriene formation by leukocyctes. The mechanism of phenylbutazone anti-inflamamtory activity is not well understood. Inhibition of biosynthesis by the peroxyl radical, which is formed by the prostaglandin hydroperoxidase, could be a new mechanism for anti-inflammatory drugs.

To examine the effect of exposure to asbestos on rat alveolar macrophage AA metabolism.

C. Publications of past 18 months

Degen, G., Wong, A., Eling, T., Barrett, J.C. and McLachlan, J. A.: Involvement of prostaglandin synthetase in the peroxidative metabolism of diethylstilbestrol in Syrian hamster embryo fibroblast cell culture. Cancer Res. 43: 992-996, 1983.

Robertson, I., Sivarajah, K., Eling, T. E. and Zeiger, E.: Activation of some aromatic amines to mutagenic products by prostaglandin endoperoxide synthetase. Cancer Res., 43: 476-480, 1983.

Marnett, L. J., Siedlik, R. H., Ocho, R., Honn, K.V., Das, M. Warnock, R., Tainer, B. and Eling, T.: Mechanism of the stimulation of prostaglandin H synthase and prostacyclin synthase by the anti-thrombotic and antimetastatic agent, Nafazatom: Mole. Pharm. In press.

Eling, T. and Ally, A.: Pulmonary biosynthesis and metabolism of prostaglandins and related substances. Environ. Health Perspect. 55: 159-168, 1984.

Henke, D., Kouzan, S. and Eling, T.E.: Analysis of leukotrienes, prostaglandins and other oxygenated metabolites of arachidonic acid by high performance liquid chromatography. Anal. Biochem. in press

Kouzan, S., Brody, A., Nettesheim, P., Eling, T.E.: Asbestos stimulates the release of arachidonic acid metabolites from alveolar macrophages. Submitted to Am. Rev. Respir. Dis.

Reed, G., Lasker, J., Eling, T. and Sivarajah, K.: Peroxidative oxidation of bilirubin: A spectrophotometric assay for prostaglandin H synthase. Prostaglandins in press.

Boyd, J. and Eling, T.: Evidence for a one-electron mechanism of 2-aminofluorene oxidation by prostaglandin H synthase and horseradish peroxidase. J. Biol. Chem. submitted

Eling, T., Mason, R. and Sivarajah, K.: The formation and subsequent decay of the aminopyrine cation radical by prostaglandin H synthase. J. Biol. Chem. submitted

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80035-08 LMB

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cooxidation of Xenobiotics by the Prostaglandin Synthetase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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SECTION

Prostaglandin Biochemistry

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long range goal of this project is to study the oxidation of chemicals to toxic metabolites by prostaglandin synthetase (PHS) and to demonstrate the significance of this system in chemically-induced toxicity or carcinogenesis. We have shown that PHS converts both polycyclic hydrocarbons and aromatic amines to mutagens as measured by bacterial tester systems. Other in vitro studies have demonstrated the formation of electrophilic metabolites that react with macromolecules. Benzo(a)pyrene-7,8-diol is metabolized to an anti-diol epoxide by PHS. We have compared PHS and NADPH-dependent metabolism in hamster trachea and human bronchial explants. In both tissues, stimulation of PHS increased anti-diol epoxide formation. We have also shown that the anti-inflammatory drug phenylbutazone is converted by PHS to a phenylbutazone peroxyl radical which can epoxidize BP-7,8-diol. This represents a new mechanism for the metabolism of chemicals by PHS. The aromatic amine carcinogen 2-aminofluorene (2-AF) is metabolized to free radical intermediates by PHS. The stable end products are azo-, nitro-fluorene and 2-aminodifluorenylamine. We have studied the formation of phenolic 2-AF adducts and obtained evidence that 2-AF is oxidized to several free radicals or free radical derived products (nitrenium ion). These radicals may not only be responsible for covalent binding to DNA but also may indeed be the proximate carcinogenic and mutagenic agents. We have also studied the formation of 2-AF DNA adducts catalyzed by PHS. Several unique 2-AF-DNA adducts were detected. Our studies indicate that PSH activates chemicals to ultimate carcinogenic metabolites which may be of importance in the initiation of tumors in extrahepatic tissue. Thus PHS is an enzyme system that, like cytochrome P-450, is important in the metabolism of xenobiotics.

A. Cooxidation of Xenobiotics by the Prostaglandin Synthetase

Nature of Problem: Substantial evidence supports the hypothesis that metabolism is a prerequisite for the initiation of tumor formation in animal tissue by carcinogens. Polycyclic aromatic hydrocarbons (PAH) and aromatic amines are oxidized to electrophilic metabolites that bind to critical tissue nucleophilic macromolecules, resulting in cell transformation. Understanding the processes of metabolism is therefore important for understanding tumor formation. This may be necessary for the early detection of new environmental carcinogens and the development of inhibitors of carcinogenesis. It is now well established that the metabolism of many chemicals to reactive intermediates is accomplished by the cytochrome P-450 mixed function oxidase system (MFO). During the oxidation of arachidonic acid (AA) to prostaglandins by prostaglandin synthetase (PHS), chemicals are also oxidized. This peroxidatic oxidation can be considered as cooxidation. PHS is ubiquitous and is particularly rich in target organs like the bladder, lung and skin which have low MFO activity. Cooxidation by prostaglandin synthetase may be an additional enzyme system for activation of chemicals.

Objectives: 1) To characterize the metabolites of carcinogens catalyzed by PHS. 2) To study the mechanism(s) of co-oxidation. 3) To relate chemical metabolism by PHS to development of chemical-induced toxicity. 4) To develop biochemical endpoints necessary to access importance of co-oxidation *in vivo*.

Experimental Approach: Metabolites are isolated and separated by HPLC and characterized by U.V. spectra, MS and NMR. In some studies direct detection of the radicals by electron spin resonance is employed. Mutagenic metabolites are detected by a modified Ames test. Initial studies are done using ram seminal vesicles microsomes or horseradish peroxidase (HRP) as a source of peroxidase. Studies proceed to using microsomes from target tissue, cells in culture or explants and eventually to whole animals. Our approach is to understand basic mechanism by developing tools that relate metabolism to toxicity, particularly under *in vivo* condition.

Recent Accomplishments: We have examined the metabolism of BP-7,8-diol in hamster tracheal explants. BP-7,8-diol was oxidized to both anti- and syn-diol epoxides. The addition of arachidonic acid stimulated the formation of anti-diol epoxide. The ratio of *anti* to *syn* increased from 2:1 to 4:1. Indomethacin reduced the arachidonic acid stimulation. The dependence on the concentration of AA was studied. At a concentration as low as 25 μm, stimulation of metabolism was observed. These results suggest endogenous AA will support oxidation to the *anti*-diol epoxide.

The addition of arachidonic acid also stimulated the formation of the *anti*-diol epoxide by human broncheal explants. However, data was more variable and stimulation of metabolism was not inhibited by indomethacin. Analysis of the arachidonic acid metabolites showed that PHS metabolites were formed by hamster tracheal explants while lipoxygenase metabolites were produced by human broncheal explants. Lipoxygenase is not inhibited by indomethacin. These data suggest hydroperoxy fatty acids supported the peroxidase mediated metabolism. We conclude that co-oxidation does occur in these tissues but is not of major importance to total metabolic activation.

The mechanism for the epoxidation of BP-7,8-diol was studied. Plenylbutazone, an anti-inflammatory agent, was found to stimulate the epoxidation reaction. Also the hydroperoxide specifically was altered such that H_2O_2 could catalyze the epoxidation. We propose that phenylbutazone is metabolized by PHS via a peroxyl radical to 4-hydroxyphenylbutazone. The peroxyl radical appears to be the epoxidizing agent. By this mechanism the peroxidase incease the oxidative capacity rather than decreasing it. Our findings suggest a new mechanism for the metabolism of chemicals by PHS.

The metabolism of benzidine and several derivatives was studied using either HRP or PHS. The model chemical, 3,5,3',5'-tetramethylbenzidine (TMB) was oxidized to a radical cation and a charge-transfer complex composed of TMB and its two electron (di-imine) oxidation products. Benzidine and other benzidine derivatives appeared to be oxidized by HRP and PHS in a similar series of reactions. The two electron oxidation products, the di-imines and a resonance structure of the nitrenium ions, are the proposed as the ultimate carcinogenic metabolites of this aromatic amine. We have also recently shown that the carcinogen 2-aminfluorene (2-AF) is metabolized by PHS. Metabolism of 2-AF was dependent on arachidonic acid or a hydroperoxide and inhibited by indomethacin. AF inhibited the peroxidatic oxidation of phenylbutazone by PHS hydroperoxidase, suggesting that 2-AF is a reducing co-factor for PHS. The metabolites of 2-AF formed by PHS were isolated and identified by UV-visible spectrophotometry, HPLC and mass spectrometry as 2-nitrofluorene, azofluorene, 2-aminodifluorenylamine (ADFA), polymer and metabolites that covalently binding to protein and DNA. Similar metabolites were produced by horseradish peroxidase while chloroperoxidase produced polymer and nitrosofluorene. We have also studied the formation of phenolic 2-AF adducts catalyzed by HRP and PHS. Two adducts were found with BHA, one in which the 2-AF nitrogen is linked to para position of BHA and a second in which the amino group if 2-ADFA is linked to para position of BHA. In both cases suggest that the reaction is facilitated by leaving of $HOCH_3$ group. These findings, together with other available evidence, indicates that 2-AF is metabolized by PHS to free radicals which are responsible for the formation of DNA and phenolic adducts. While nitrenium ion formation cannot be excluded the N-hydroxlyamine can be eliminated as a possible intermediate.

We have also studied the formation of 2-AF DNA adducts catalyzed by HRP and PHS. In addition to small amount of guanine adduct formed from a nitrenium ion, three unique adducts were observed. These will serve a useful indices for 2-AF co-oxidation <u>in vivo</u>.

The oxidation of aminopyrine was used as a tool for studying PHS catalyzed N-demethylation. Aminopyrine is oxidized to a cation free radical by the hydroperoxidase component of purified PHS. The stoichiometry between the amount of 15-hydroperoxy AA reduced to aminopyrine oxidized was 1:2, in agreement with a peroxidase mediated reaction. The decay of the radical was found to obey second order kinetics which is consistent with our previous proposal of a disproportionation mechanism for N-demethylation.

B. Plans for subsquent year:

1. We will study the epoxidation of BP-7,8-diol in isolated mouse skin cell. Mouse skin is a useful model of BP induced tumor formation. We will use (+) BP-7,8-diol as a biochemical marker for co-oxidation since the (+)-isomer is converted by PHS to anti diolperoxide while P-450 converts the (+)-isomer to the syn diolperoxide. Using this model system we will also investigate the role of lipid peroxidation in the metabolism of BP-7,8-diol.

2. We will characterize the PHS dependent 2-AF-DNA adducts. Once characterized these adducts will be used to determine if the co-oxidation pathway is operative in bladder cells and in the dog bladder (in vivo). The post labeling method for DNA adducts is being developed in collaboration with Dr. Anderson.

3. We will continue to characterize the metabolites of amines catalyzed by peroxidase. We will shift our focus to 2-napthylamine since peroxidase mediated metabolism appears to be different from 2-AF. We need to understand why these differences in metabolites occur.

4. We will attempt to study the formation of radicals derived from various chemicals and carcinogens by PHS using intact cells. In only a few cases have free radicals in cells been detected. These studies will be done by Dr. Schrieber in collaboration with Dr. Mason.

5. We will study the formation of styrene GSH adducts catalyzed by PHS. Preliminary evidence indicate a unique mechanism for the formation of these conjugates. This work is in collaboration with Dr. Bend.

C. Publications of past 18 months

Boyd, J., Harvan, D., and Eling, T.E.: The oxidation of 2-aminofluorene by prostaglandin endoperoxide synthetase. J. Biol. Chem. 258: 8246-8254, 1983.

Eling, T.E., Boyd, J., Reed, G., Mason, R., and Sivarajah, K.: Xenobiotic metabolism by prostaglandin endoperoxide synthetase. Drug Metab. Review 14: 1203-1253, 1983.

Josephy, P. D., Eling, T. and Mason, R.: Cooxidation of benzidine by prostaglandin synthetase and comparison with the action of horseradish peroxidase. J. Biol. Chem. 258: 5561-5569, 1983.

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RALTECH SCIENTIFIC SERVICES - Madison, Wisconsin 60616
(NIH-NO1-ES-7-2141)

TITLE: Analysis of PCB's and DDE in Human Body Fluids and Tissue

CONTRACTOR'S PROJECT DIRECTOR: D.L. Hughes

PROJECT OFFICER (NIEHS): J.D. McKinney, Ph.D., Supervisory Research Chemist, LMB

DATE CONTRACT INITIATED: September 30, 1977

CURRENT LEVEL (1 year): 0

PROJECT DESCRIPTION

OBJECTIVES: Analysis of 1000 to 1200 samples per year of breast mild, formula, blood serum and placental tissue for polychlorinated biphenyls (PCB's) and 1,1-bis(p-chlorophenyl)-2,2-dichloroethane (DDE). The desired detection thresholds range from 0.5 to 50 ppb depending on the type of sample.

METHODS EMPLOYED: Gas liquid electron capture chromatography (EC-GC) and usual sample preparation, clean-up, extraction and lipid determination techniques.

MAJOR FINDINGS OF PROPOSED COURSE: Using the methods developed previously on the contract, a total of over 6000 milk, milk substitutes, blood serum or placenta tissue have been analyzed for PCB and DDE content. Final analyses are being completed, detailed reports prepared, and samples of unused fluids and tissues and their extracts return shipped to the Institute. Method paper for PCBs and DDE analyses of milk, milk substitutes and serum has been accepted for publication. Interpretation of the results and correlation with biological findings are in progress and will be reported elsewhere. This project has been completed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The possible effect of transplacental and breast milk transfer of environmental contaminants from mothrs to babies is an important and recurring epidemiological question. It has been shown that environmental contamination of breast milk occurs in the United States. Organohalogen pesticides and the polychlorinated biphenyls (PCB's) are widespread contaminants of breast milk. This study provides an integral part of an overall study of the possible widespread contamination of breast milk by environmental contaminants such as PCB's and their effects on infant development and health.

SCIENCE APPLICATIONS, INC. - La Jolla, Clifornia 92038
(NIH-NO1-ES-8-2105)

TITLE: Analysis of Total Organic Chlorine and Bromine Residues in Human Body Fluids and Tissues

CONTRACTOR'S PROJECT DIRECTOR: J. Reed

PROJECT OFFICER (NIEHS): J.D. McKinney, Ph.D., Supervisor Research Chemist, LMB

DATE CONTRACT INITIATED: December 20, 1977

CURRENT LEVEL (2 years): 0

PROJECT DESCRIPTION

OBJECTIVES: Analysis of 1500 to 2000 samples per year of brease milk, formula, blood serum, and tissue for total organic chlorine (TOCl) and bromine (TOBr) of whole fluid or wet tissue or for total soluble organic chlorine (TSOCl) and bromine (TSOBr) content of portions of extractable lipids. The desired detection thresholds range from 5-20 ng chlorine and 0.1-15 ng bromine/gm milk.

METHODS EMPLOYED: BioGel P-2 desalting followed by standard methods of sample preparation for neutron activation analysis (NAA).

MAJOR FINDINGS AND PROPOSED COURSE: Using methods developed previously on the contract, over 4000 milk and serum samples have been analyzed for TOCl and TOBr, over 1000 milk and milk substitutes for total chlorine and bromine, and about 500 placenta samples for total chlorine and bromine. The contract is in a no cost extension phase to complete detailed reporting writing. The methods for TOCl and TOBr in milk and serum have been published. This project has been completed.

SIGNIFICANCE OT BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Obtainment of accurate and reliable results that lead to meaningful interpretations of the transfer of PCB's and DDE from mother to child through placental tissue membranes or through breast milk requires the separation of organic bound chloride and bromine from inorganic chlorides and bromides prior to neutron activation analysis for TOCl, TOBr, TSOCl and TSOBr. This study integrates contract NIH-NO1-ES-7-2141 to provide a mass balance which indicates whether all the halides are accounted for by the PCB's and DDE. In this manner, the results of the contract for the analysis of PCB's and DDE in human body fluids and tissues can be evaluated. This study will help resolve the important epidemiological effects of possible transplacental and breast milk transfer of environmental contaminants from mothers to babies in the United States.

LABORATORY OF PHARMACOLOGY

LABORATORY OF PHARMACOLOGY
Summary Statement

The Laboratory of Pharmacology carries out research to elucidate the relationships between the transformation and translocation of chemicals and toxicity in various target organs and cells of the body. A multidisciplinary approach is used in these investigations: pharmacologists, biochemists, chemists, pathologists, physiologists, toxicologists, and statisticians participate. This laboratory provides a central focus at NIEHS for using pharmacological and pharmacokinetic concepts to characterize, in detail, the mechanisms by which environmental contaminants exert biological effects. It plans and conducts studies 1) to determine the metabolic basis for selective/specific damage to certain organs and cell types which is characteristic of some toxins, 2) to elucidate the mechanisms whereby chemicals with hormonal activity alter the functions of organ systems at the molecular level, 3) to determine the role of membrane structure and function in excretion and toxicity of pollutants, 4) to correlate organ and cell structure with function both before and after exposure to chemicals, and 5) to identify sensitive biochemical, pharmacological, physiological and pathological indicators of target organ/cell toxicity useful for the early detection and prediction of toxicity in experimental animals and humans. The Laboratory of Pharmacology also serves as a focal point within NIEHS, NIH and DHHS for marine and freshwater biomedical research. In this context we are especially interested in possible direct impact on human health by comtaminants present in the aquatic environment (including drinking water) and accumulated by aquatic animals. Presently, the Laboratory of Pharmacology contains the Molecular and Comparative Pharmacology and Cell Pharmacology sections.

A. Molecular and Comparative Pharmacology Section (Head: Dr. J.R. Bend)
The overall activity of this group can be described as an integrated, multifaceted effort concerned with understanding the role of chemical metabolism, transport and excretion in the mediation of toxicity such as overt tissue damage, or more subtle effects such as carcinogenesis, mutagenesis and teratogenesis.

For many chemicals, the processes of metabolism are means of both activation and inactivation and the relative activities of these pathways/steps, as well as their location in different cells, and parts of cells, are most critical to the particular outcome of exposure to any given chemical. That these processes of metabolic activation and inactivation are themselves often controlled by genetics, as well as being affected by age, sex, disease and environment, further complicate the understanding of their role in the effects of any given chemical in any given tissue or animal species or individual of that species at any specific time of exposure.

Major emphasis is currently focused on toxication-detoxication systems, transport and excretory mechanisms and membrane toxicity.

Another major purpose of this section is to serve as a national focus for an aquatic pharmacology/toxicology program -- to promote awareness of and use of such aquatic species and systems in better understanding human disease and contributions of pollution to such disease.

The collaborative efforts of this group demonstrate both its desire to share expertise where possible as well as to make use of the many opportunities for introducing more powerful and new approaches in this research area of chemical metabolism as related to toxicity. This group also interacts very closely with the Cell Pharmacology Section of Dr. J.R. Fouts.

Recent Accomplishments:

1. Dr. Bend's laboratory:

a. Subsequent to the development of an HPLC system that separates the four diastereomeric glutathione adducts of benzo[a]pyrene 4,5-oxide (BPO), the stereoselectivity of glutathione transferases with BPO as a substrate was studied. Sufficient variation in stereoselectivity of purified transferases from various sources suggest that this parameter may be useful for the functional characterization of these enzymes.

b. The stereoselectivity of the cytochrome P-450-dependent oxidation of styrene to R- or S-styrene 7,8-oxide (SO) was studied. All P-450 isozymes investigated were found to catalyze this reaction and SO was the predominant product formed in each case; moreover, the stereoselectivity of the reaction varied considerably and was isozyme dependent.

c. N-Benzyl-1-aminobenzotriozole was synthesized, chemically characterized and shown to be a very effective suicide inhibitor of cytochrome P-450, form 2, in rabbit lung microsomes although it had no suicidal activity for P-450, form 5. The data demonstrated that P-450 isozyme selectivity can be introduced into the 1-aminobenzotriozole molecule by N-substitution and that such substitution does not diminish the suicidal pathway of these substrates for inactivation of responsive isozymes.

2. Dr. Philpot's laboratory

a. Highly sensitive techniques were developed for quantitation of several isozymes of cytochrome P-450.

b. Analogous differences between the effects of exogenous chemicals on hepatic and pulmonary P-450 systems were found in all species examined (3 strains of rats, mouse, guinea pig, hamster and rabbit).

c. The microsomal fraction of rabbit bladder is highly active in cytochrome P-450-dependent N-oxidation of 2-aminofluorene but not 2-acetylaminofluorene. The metabolism of 2-acetylaminofluorene to reactive products appears to be initiated by deacetylation to 2-aminofluorene in rabbit bladder.

3. Dr. Negishi's laboratory:

a. Female specific testosterone 15α-hydroxylase ($P\text{-}450_{15\alpha}$) was purified from female 129/J mice.

b. Male specific testosterone 16α-hydroxylase ($P\text{-}450_{16\alpha}$) was purified from male 129/J mice. $P\text{-}450_{15\alpha}$ and $P\text{-}450_{16\alpha}$ are minor components of total microsomal cytochrome P-450 in untreated mouse liver (about 2%); the purifications were carried out based on specific hydroxylation activity in fractions eluted from columns.

c. Two cDNAs encoding cytochrome P-450s which are associated with the Ah-locus were isolated and sequenced. The Ah-locus regulates cytochrome P-450 induction by polycyclic aromatic hydrocarbons.

4. Dr. Pritchard's laboratory:

a. Luminal membranes from rat kidney were shown to possess two separate carriers for $SO_4^{=}$, an $Na^+/SO_4^{=}$ cotransport system and an Na^+ independent, SITS sensitive anion exchanger.

b. Rat kidney basolateral membranes were demonstrated to transport sulfate and organic anions via anion exchange. Exchange may be further stimulated by imposition of an Na^+ gradient, raising the possibility that directional control of sulfate transport may be achieved by controlling the coupling of exchange to Na^+.

c. Both sulfate, an inorganic anion, and L-lysine, an organic cation, are transported by pH gradient dependent mechanisms at the plasma membrane. Based on this knowledge, the pesticide, pentachlorophenol which dissipates pH gradients by acting as a proton conducting ionophore was shown to be an effective inhibitor of renal transport of each of these solutes.

5. Dr. Fowler's laboratory:

a. High affinity renal cytosolic lead-binding proteins were shown to mediate the intranuclear movement of Pb^{2+} in a time- and temperature-dependent manner thereby regulating the interaction of lead (Pb^{2+}) with DNA and affecting subsequent genotoxic effects of lead.

b. High affinity renal cytosolic lead-binding proteins were shown to markedly influence the sensitivity of δ-aminolevulinic acid dehydratase to lead inhibition in a concentration-dependent manner.

c. Oyster cadmium-binding proteins (CdBP) were shown to possess a variable number of SH groups/Cd atom in contrast with the stable SH:metal ratio of the metallothionein binding sites which results in the unusual circular dichroic properties of the site.

6. Dr. Anderson's laboratory:

a. The *in vivo* formation of benzo(a)pyrene (BP) metabolite-DNA adducts were characterized in several cell types of lung and liver. Alveolar macrophages, alveolar type II cells and Clara cells isolated from lung and hepatocytes and non-parenchymal cells isolated from liver of rabbits treated with ^{3}H-BP contained similar levels of (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP-deoxyguanine.

b. An *in vivo* - *in vitro* method was developed to measure unscheduled DNA synthesis (UDS) in liver and lung. The sensitivity of this method is at least 10-fold greater than the *in vivo* approach previously described.

c. A nucleoid assay system is being developed to quantitate carcinogen-induced DNA damage at various times after chemical administration.

B. Cell Pharmacology Section (Head: Dr. J.R. Fouts)

This group investigates the localization of drug and pollutant metabolizing enzyme systems in tissues that serve as interfaces with the environment (e.g., lung, skin and gut). This research group is also investigating factors which affect chemical-metabolizing systems, the development of these systems in the perinatal period, and on species differences in these systems. Cell types are isolated from these organs and enriched by elutriation and centrifugation techniques. The contribution of the metabolic systems in individual cells to target organ and cell toxicity is evaluated.

Assay systems are being developed so that both oxidation and conjugation pathways of chemical metabolism can be quantitated in single cells. Such procedures will eventually be extended to other systems, including cells in culture. The scientists in this section frequently collaborate with those in the Molecular and Comparaive Pharmacology Section.

Another focus of interest is intestinal function and toxicology at the cellular, subcellular and molecular levels. A better understanding of the basic biochemistry, physiology and pharmacology of the normal intestine should permit greater appreciation for the unique roles of this organ in absorption and metabolism. In addition, this better understanding of normal function may lead to better methods for the detection of dysfunction and toxicity.

Recent Accomplishments:

1. Dr. Fouts' laboratory:

a. Lung cells: Enzyme differences in major lung cell types were investigated by both selected enzyme activities (this year especially lipid and steroid metabolisms, P-450 isozymes) and immunochemical (antibodies to specific P-450 isozymes) assays. Also devised methods for isolating another major lung cell type -- the ciliated cell -- in good yields and high purity; this allows study of its enzyme composition in comparison with other lung cell types (Clara and type II cells). We have also shown that the possible "degradation" of P-450 during cell preparation (especially in Clara cell and as shown by the presence of P-450 peptides in Western blots of freshly isolated cells) can be reduced by changes in preparative procedures (especially adding anti-proteases, antioxidants). However, no technique is yet developed which eliminates these peptides and indeed these are also seen in the best preparations of hepatocytes (as prepared by standard methods) as well.

b. Skin cells: In collaboration with Drs. Coomes and Eling/Henke we have finished the first phase of the characterization of isolated cells from hairless mice. Cells in different layers of the skin have markedly different xenobiotic and lipid metabolisms. The work with arachidonic acid metabolism mirrors that with xenobiotics -- more metabolism with more differentiated cells and especially by sebaceous cells.

c. Liver cells: With Drs. Klinger and Maronpot we have shown that the development of hepatic lobular differentiation (centrilobular xenobiotic metabolism is greater than periportal for several pathways) occurs after birth -- in the immediate perinatal period the metabolisms of several chemicals are not different in these two areas of the lobule in the rat.

2. Dr. Schiller's laboratory:

a. A (sub)strain difference in sensitivity to TCDD (single oral dose in corn oil) was determined for 4 (sub)strains of rats.

b. A single oral dose of TCDD was shown to induce impaired lipid assimilation in rats, i.e., mesenteric lymph lipoproteins are larger and have a decreased apo AI/apo E.

c. Similarity in sensitivity to TCDD and to altered carbohydrate/lipid parameters in C57 and DBA mice suggest a common mechanism of lethality. In both rats and mice, there was no dose related correlation between weight loss and food consumption.

D. Collaborative Efforts

As can be seen from the individual project descriptions, scientists in the Laboratory of Pharmacology are involved in many activities and collaborative research efforts with scientists here as NIEHS and elsewhere.

Examples of collaborative programs outside of NIEHS for the senior scientists are: Dr. Bend with Dr. Bengt Mannervik of the University of Stockholm, and with Drs. Mike Meredith and Fred Guengerich, Vanderbilt University; Dr. Fouts with Dr. Wolfgang Klinger of the University of Jena, Dr. Ken Jones, University of

Toronto and Dr. Maggie Coomes, Howard University; Dr. Philpot with Dr. Eric Johnson of Scripps Clinic and Research Foundation, Drs. Paul Thomas and Wayne Levin, Hoffmann-LaRoche, and Dr. Ed Bresnick, Eppley Institute; Dr. Pritchard with Drs. Paul Linser and Margaret James, University of Florida, Gainsville and Dr. A.R. Krall, Medical University of South Carolina; Dr. Fowler with Dr. David Petering, University of Wisconsin, Milwaukee and Dr. Dennis Winge, University of Utah; Dr. Negishi with Dr. J.E. Shively, City of Hope, California and Dr. Anderson with Dr. Stuart Aaronson of the National Cancer Institute, Dr. Jim Swenberg of the Chemical Industry Institute of Toxicology and Dr. Steve Strom of Duke University.

The collaborative efforts are cited only to show the extensive interactions of this Laboratory with groups outside NIEHS. In addition to these contacts, those with faculty and researchers in the Triangle area are too numerous to document, but add strength to our activities, peer reviews (in terms of seminars, discussions, exchange of students) and opportunities for advice, new techniques, and short courses not only for our staff but for members of the other institutions as well.

E. Personnel

A major change occurred in the Laboratory of Pharmacology (LP) during August of this year in that Dr. Pritchard's program moved from the C.V. Whitney Marine Laboratory in Florida to NIEHS, Research Triangle Park. An addition to LP during FY'84 was Dr. Steven Reynolds (Staff Fellow with Dr. Anderson). Losses included Dr. George Lucier and his research group (Dr. Richard C. Rumbaugh, Dr. Calliopi Mastry, Ms. Otelia McDaniel, Mr. Zadock McCoy, Ms. Tracy Sloop, Ms. Diane B. Campen and Dr. Alison Vickers) who transferred to the Biometry and Risk Assessment Program. Others leaving LP were Dr. Soon-Ho Lee and Dr. Gaylen Neufeld (experts with Dr. Pritchard), Ms. Marian Nolan (a technician with Dr. Pritchard), Dr. Mahmooda Kulkarni (a Staff Fellow with Dr. Anderson), Dr. Joseph Rosenior (a Visiting Fellow with Dr. Anderson), Dr. Jean-Paul Bogaert (a Guest Worker with Drs. Schiller and Lucier) and Dr. Chon Shoaf (a Guest Worker with Dr. Schiller).

F. Other Activities

Dr. J.R. Bend: Adjunct Professor, Department of Entomology, North Carolina State University, Raleigh; Member, Toxicology Advisory Committee, North Carolina State University; Adjunct Associate Professor, Curriculum in Toxicology, School of Medicine, University of North Carolina; Member, Editorial Advisory Board for Drug Metabolism and Disposition and Board of Editors, Environmental Health Perspectives; Associate Managing Editor (U.S.A.) for Chemico-Biological Interactions; Associate Editor, Reviews in Biochemical Toxicology; Councilor, Section on "Mechanisms of Toxicology", Society of Toxicology; Coordinator for "Special Topics in Toxicology", a graduate couse given in Research Triangle Park for students at Duke, North Carolina State University and University of North Carolina; served on graduate student committees at North Carolina State University and University of North Carolina; Member, Steering Committee for Toxicokinetics Section, WHO-sponsored International Program of Chemical Safety; Presented research seminars at Vanderbilt University, Nashville and at a dinner meeting of the Delaware Valley Drug Metabolism Discussion Group, Philadelphia.

Dr. J.R. Fouts: Adjunct Professor of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill; Adjunct Professor of Toxicology, Department of Entomology, School of Life Sciences, North Carolina State

University at Raleigh; Member, Toxicology Advisory Committee, Faculty of Toxicology, North Carolina State University; Associate Editor, Pharmacological Reviews; Editorial Board of Xenobiotica, Pharmacology, Journal of Toxicology and Environmental Health.

Dr. R.M. Philpot: Adjunct Professor, Department of Entomology, North Carolina State University, Raleigh; Member, Toxicology Advisory Committee, North Carolina State University; Associate Managing Editor (U.S.A.) Chemico-Biological Interactions; Associate Editor Reviews in Biochemical Toxicology; Member, Editoral Board Molecular Pharmacology; Member, Executive Committee of the Drug Metabolism Division, American Society for Pharmacology and Experimental Therapeutics; Invited symposium speaker, IUPHAR International Congress of Pharmacology, July 29 - August 3, London and 6th International Symposium on Microsomes and Drug Oxidations, August 5-10, Brighton, UK.

Dr. John B. Pritchard: Adjunct Associate Professor, Department of Pharmacology, University of Florida School of Medicine, Gainesville; Invited speaker, Pharmacology Colloquium, University of California at Santa Barbara; Presented research seminars at Duke University, University of Arizona at Tucson, University of Texas Medical Center at San Antonio, and the University of Florida at Gainsville.

Dr. M. Negishi: Adjunct Associate Professor, School of Veterinary Medicine, North Carolina State University.

Dr. B.A. Fowler: Adjunct Associate Professor, Department of Pathology and Toxicology Curriculum, University of North Carolina, Chapel Hill; Member, Editorial Boards Chemico-Biological Interactions and Environmental Health Perspectives; Invited participant at symposium on Pollution and Physiology of Marine Organisms; Discussion leader, Gordon Conference; Co-moderator Metal Speciation in Systems under Stress Working Group for Dahlem Conference on The Importance of Chemical Speciation in Environmental Processes; Conference Chairman, NIEHS International Symposium on The Biochemistry of High Affinity Metal-Binding Proteins in Non-Mammalian Species; Invited to present seminars at the Department of Pathology, University of Washington, and Department of Pharmacology, University of Nebraska Medical Center; Course Director, Path 150/Tox 150, University of North Carolina School of Medicine.

Dr. C.M. Schiller: Adjunct Associate Professor, Department of Biochemistry and Nutrition, School of Medicine, University of North Carolina, Chapel Hill; Member of the Faculty of the Graduate Curriculum in Toxicology, University of North Carolina, Chapel Hill; Liaison member, U.S.-EPA Toxic Substances Subcommittee, Science Advisory Board, Washington, D.C.; Member, Digestive Diseases Coordinating Committee, Bethesda, MD; Alternate Member, Nutrition Coordinating Committee, Bethesda, MD; Lecturer in graduate-level courses in Biochemical Toxicology at the University of North Carolina, Chapel Hill; Graduate advisor of students from the Department of Biochemistry and Nutrition and the Curriculum in Toxicology, University of North Carolina, Chapel Hill; Sponsor of NIH Postdoctoral Fellows in Toxicology Training Program; Sponsor of ASBC Summer Fellow in High School Science Teacher Program.

Dr. M.W. Anderson: Adjunct Associate Professor, North Carolina State University, Biomathematics and Toxicology Department; Editorial Board Member, Environmental Health Perspectives; Invited speaker at Symposium sponsored by the

American Chemical Society and at an international meeting titled "DNA Adducts: Dosimeter to Monitor Human Exposure to Environmental Mutagens and Carcinogens"; Directs graduate student at North Carolina State University.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 35005-05 L

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

*Carcinogen-Induced DNA Damage and Repair (New title)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Marshall W. Anderson	Mathematician	LP	NIEHS
Other:	Dr. Felix Romagna	Visiting Fellow	LP	NIEHS
	Dr. Steve Belinsky	NRS Fellow	LP	NIEHS
	Dr. Steven Reynolds	Staff Fellow	LP	NIEHS
	Ms. Jill Stowers	"P" Appointment	LP	NIEHS

COOPERATING UNITS *(if any)*

Drs. John Bend and Richard Philpot, Laboratory of Pharmacology; Dr. Steve Strom, Duke University; Dr. Robert Maronpot, National Toxicology Program; and Dr. Stewart Aaronson, National Cancer Institute

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There is compelling evidence that many mutagens and carcinogens are able to react with cellular DNA either directly or following metabolic formation of reactive products. If DNA replication proceeds on such a modified template before altered bases or nucleotides are removed by enzymic repair processes, the mutations can be genetically fixed. Thus, the extent of carcinogen-induced promutagenic DNA damage and the capacity of cells to repair such damage represent critical events in the initiation of carcinogenesis. We are studying the in vivo formation and repair of carcinogen metabolite-DNA adducts in tissues and cells that are susceptible or resistant to carcinogen-induced neoplasia. We are concerned with the effects of dose of carcinogen on the amounts and types of adducts formed and on the subsequent repair of these adducts. Our studies on the in vivo formation and repair of benzo(a)pyrene (BP) metabolite-DNA adducts in a variety of tissues and cell types emphasize the possibility that long-term exposure to low levels of BP could result in the accumulation of BP-DNA adducts in cells which have slow turnover rates. Even if environmental exposure to BP (and other polycyclic aromatic hydrocarbons) is too small to induce neoplasia, the persistence of BP-DNA adducts may produce aberration in transcripts of genetic information in various cell types and lead to other toxic effects. We have developed several techniques to study in vivo repair of carcinogen-induced DNA damage. 1) An in vivo - in vitro to measure unscheduled DNA synthesis and 2) a nucleoid assay to detect carcinogen-induced changes in DNA superhelicity as well as study of time-dependent repair of such damages.

(*Formerly titled "Pharmacokinetic Considerations in the Formation and Repair of Carcinogen-DNA Adducts)

PROJECT DESCRIPTION

A. Carcinogen-Induced DNA Damage and Repair

Nature of Problem: There is compelling evidence that many mutagens and carcinogens are able to react with cellular DNA either directly or following metabolic formation of reactive adducts. If DNA replication proceeds on such a modified template before altered bases or nucleotides are removed by enzymic repair processes, the mutations can be genetically fixed. Thus, the extent of carcinogen-induced promutagenic DNA damage and the capacity of cells to repair such damage represent critical events in the initiation of carcinogenesis. Although there have been numerous studies of chemical-induced DNA damage and repair processes in in vitro model systems, in vivo studies for certain classes of carcinogens such as polycyclic aromatic hydrocarbons (PAH) have been neglected. An analysis of the factors that determine the initial extent of in vivo carcinogen-DNA adduct formation and the capacities of the various repair processes in target and nontarget tissues (cells) should provide relevant information about tissue and cellular selectivity in chemical-mediated neoplasia and other toxicological responses.

A problem of practical importance in environmental toxicology is to predict the potential chemical insult to man from high dose toxicology data in laboratory animals. Thus, both low dose and species-to-species extrapolation of toxicology data are involved in the risk assessment of human exposure to chemicals. Carcinogen-DNA adduct levels and repair rates of the adducts can usually be measured at much lower doses of the carcinogens than the doses used in bioassay studies. The potential use of the amount and persistence of carcinogen-DNA adducts formed in the target cell as a measure of the effective dose of a carcinogen should help in the low dose and species-to-species extrapolation of carcinogenic data.

Objectives in near term:

1. We will continue to examine the in vivo formation and subsequent repair of carcinogen-DNA adducts in both target and non-target cells. Several hypothesis will be further tested: a) Does initial adduct levels and their persistence in target cells contribute an effective measure of the biological dose of the carcinogen , and b) does carcinogen-induced DNA damage in non-target cells impose an additional toxicological risk

2. We will examine possible interactions between carcinogens and the damage they inflict on DNA. We will test the hypothesis that DNA damage induced by one carcinogen inhibits the repair of damage induced by another carcinogen.

3. To explore the mechanism(s) by which BPDEI- anbd II-DNA adducts are formed in tissues such as muscle and brain. Previous results suggest that BP metabolites (BPDEI and II) may be formed in tissues such as lung and liver and then transported by carrier protein to other tissues such as muscle and brain where they bind to DNA and protein. This hypothesis will be tested.

4. To continue to explore the mechanism(s) by which BHA and aryl hydrocarbon hydroxylase (AHH) inducers inhibit BP-induced neoplasia. The relationship between inhibition of BP metabolite-DNA adduct formation and induction or repression of the synthesis of specific forms of cytochrome P-450 will be examined as a function of time after TCDD administration. This will be the first step in testing the hypothesis that changes in the concentrations of specific forms of cytochrome P-450 are related to the anticarcinogenesis mode of action of AHH inducers.

Experimental design:

1. Formation and persistence of carcinogen-DNA adducts:

Animals were treated with various doses of radiolabeled carcinogen and then sacrificed at selected time points. In studies with BHA and AHH inducers, animals were first treated with the inhibitors at doses known to inhibit BP-induced neoplasia. DNA was isolated from tissue by phenolic extraction plus hydroxyapatite chromatography. Isolated DNA was enzymatically digested to individual nucleosides. High pressure liquid chromatography (HPLC) procedures were developed to analyze for carcinogen metabolite-deoxynucleoside adducts. A technique developed by Randerath and coworkers to analyze for adducts by ^{32}P-postlabeling is being explored as an alternative method to quantitate adducts. No studies on the efficiency of this process have been published. Tritium-labeled BPDEI-dGuo adducts are being used to determine the efficiency of the ^{32}P-postlabeling method. Also, in order for the technique to be practical for examining adducts which occur at low frequency, the unmodified nucleotides must be removed before labeling with ^{32}P. We feel this technique has great potential for examining *in vivo* carcinogen-DNA adducts in individual cell types.

2. Examination of in vivo DNA repair processes:

Carcinogen-induced unscheduled DNA synthesis (UDS) was examined by separating *de novo* DNA synthesis from repair synthesis by alkaline CsCl gradient(s). BrDU pellets were implanted subcutaneously in animals to label the replicating DNA. ^{3}H-thymidine (i.p.) was used to label the DNA undergoing repair synthesis. An *in vivo - in vitro* method to quantitate UDS is also being developed. Animals are treatd with carcinogens and then sacrificed at various times. Several hours before sacrifice, a BrDU pellet is implanted subcutaneously. Tissue slices are prepared and incubated in Williams Medium containing fetal calf serum (10%), L-glutamine (2 mm), BrDU (5×10^{-5}M), gentamicin (1 µg/ml) and ^{3}H-cytidine (5 µC/ml). Purified DNA is then separated into newly replicated and non-replicated DNA by ultracentrifugation in alkaline CsCl gradients. UDS is measured in the non-replicated DNA. In the nucleoid assay system, to detect carcinogen-induced changes in DNA superhelicity, organs or isolated cell types are homogenized in a solution containing 2-mercaptoethanol and nucleoids prepared with a modified procedure previously developed by Cook and Brazell. The sedimentation of the nucleoids are examined in neutral sucrose gradients. Changes in sedimentation distances reflect changes in superhelicity.

Recent accomplishments:

1. We have characterized the in vivo formation of BP metabolite-DNA adducts in a variety of target and non-target tissues of mice and rabbits. Tissues included were lung, liver, forestomach, colon, kidney, muscle, and brain. The major adduct identified in each tissue was the (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP (BPDEI)-deoxyguanosine adduct. A 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydro-BP (BPDEII)-deoxyguanosine adduct, a (-)-BPDEI-deoxyguanosine adduct, and an unidentified adduct were also observed. The adduct levels are unexpectedly similar in all the tissues examined from the same BP-treated animal. For example, the BPDEI-DNA adduct levels in muscle and brain of mice were approximately 50% of those in lung and liver at each oral BP dose used. We have also examined adduct levels formed in vivo in several cell types of lung and liver. Macrophages, type II cells, and Clara cells from lung and hepatocytes and nonparenchymal cells from liver were isolated from BP-treated animals. BPDEI-deoxyguanosine adduct was observed in each cell type and, moreover, the levels were similar in various cell types. There are several explanations for the in vivo binding of BP metabolites to DNA of most tissues (cells). It is possible that the oxidative metabolism of BP in each of the examined tissues is sufficient to account for the observed DNA binding. However, there is obviously no correlation between monooxygenase activity and DNA binding. It is also possible that BP is metabolized in tissues such as lung and liver and then BPDEI and II are transported to tissues such as brain and muscle where they bind to DNA. In any case, exposure of animals to BP results in the binding of BP metabolites to the DNA of all tissues examined and, moreover, the relative binding levels among the tissues in the same BP-exposed animals are similar. Since this observation is true for each animal species examined, for each BP dose used and for each route of administration employed, and since in vitro incubation of human tissue with BP results in the formation of BPDE-DNA adducts, it is very probable that DNA in many human tissues is continuously damaged by exposure to this ubiquitous environmental carcinogen and other PAHs..

2. The formation of BP metabolite-DNA adducts was examined as a function of BP dose ranging from 2 to 1351 μmol/kg. The dose-response relationship for BPDEI-dGuo adducts approached linearity at low doses and thus, there does not appear to be a threshold dose below which binding of BP metabolites to DNA does not occur.

3. We have examined the mechanism(s) by which the phenolic antioxidant BHA inhibits BP-induced neoplasia. The results of several studies have shown that treatment of mice with BHA inhibits adduct formation in lung and forestomach over a large BP dose range, 2 to 1351 μmol/kg. These results are consistent with the hypothesis that BHA inhibits BPDE adduct formation by altering the oxidation of BP-7,8-diol.

4. Aspirin and indomethacin, inhibitors of prostaglandin synthetase (PG), had no effect on the in vivo formation of BP metabolite-DNA adducts in lung, liver and forestomach of A/HeJ mice after an oral dose of ^{3}H-BP. Doses of aspirin and indomethacin that inhibit pulmonary PG were used. We also showed that treatment of A/HeJ mice with aspirin had no effect on BP-induced pulmonary ademonas. Thus, PG is probably not involved in the in vivo metabolism of BP, at least as it relates to DNA alkylation.

5. The persistence of benzo(a)pyrene (BP) metabolite-DNA adducts has been studied in lung and liver of A/HeJ and C57BL/6J mice after a dose of BP (6 mg/mouse) that induces pulmonary adenomas in A/HeJ mice but not in C57BL/6J mice. The disappearance of BPDEI adduct in A/HeJ mice followed first order kinetics over the time period examined, with a half-life of 18 and 9 days in lung and liver, respectively. The decay of this adduct in C57BL/6J mice was biphasic in both tissues. These results suggest that the tissue specificity for BP-induced neoplasia in A/HeJ mice may be related to the relative persistence of adducts and high cell turnover rates in lung. In contrast, the results on formation and persistence of adducts and cell turnover do not provide an explanation for the strain difference in susceptibility to BP-induced pulmonary adenomas. It was also shown that the rates of removal of BP metabolite-DNA adducts in A/HeJ mice are not significantly different at a 500-fold lower BP dose.

6. We examined in vivo DNA repair synthesis in liver and lung of A/HeJ mice exposed to BP and 4-nitroquinoline-1-oxide (4-NQO). To differentiate between the removal of carcinogen metabolite-DNA adducts due to cell turnover or DNA repair, we measured UDS in the non-replicating DNA fraction. BP induced UDS in liver at oral doses of 0.3 and 3.0 mg per mouse, whereas UDS was not detected in the lung. Our technique was capable of detecting UDS in lung since 4-NQO did not induce UDS in lung. This is the first report of UDS in vivo following BP administration (or any PAH). These and previous results clearly demonstrate that in vivo disappearance of adducts cannot necessarily be equated with nucleotide excision repair. The presence of excision repair in the liver and the liver's low rate of DNA synthesis provide an explanation for the relative resistance of this tissue to carcinogenesis by BP, since under altered conditions of DNA replication following hepatectomy, tumors can be induced by PAH treatment. In contrast to the liver, the lack of excision repair of BP metabolite-DNA adducts in lung, and the relatively high rate of DNA turnover in lung are favorble conditions for the fixation of promutagenic lesions.

7. Our results and thos of Kleihues et al. emphasize the possibility that long-term exposure to even low levels of BP could result in accumulation of PAH metabolite-DNA adducts in cells which have slow turnover rates. Even if environmental exposure to PAH is too small to induce neoplasia, the persistence of DNA adducts may produce abberations in transcripts of genetic information in various organs and lead to other toxic effects.

8. Concentrations of carcinogen metabolite-DNA adducts and/or their persistence do not adequately explain the differences in organ and species susceptibility to chemical-induced neoplasia. However, this does not detract from the use of adduct levels as a measure of the effective biological dose of the carcinogen in a known target tissue for purposes of low-dose extrapolation of carcinogenic data.

B. Plans for subsequent year

1. Although the in vivo persistence of carcinogen-DNA adducts has been examined in some detail, there have only been a few attempts to measure DNA repair synthesis in animals. We will continue to develop several techniques to study

in vivo repair of carcinogen-induced DNA damage. One involves an in vitro - in vivo approach to examine unscheduled DNA synthesis. Another involves an in vivo - in vitro system using the nucleoid assay to detect carcinogen-induced changes in DNA superhelicity and the time-dependent repair of such damage. The sensitivity for quantitation of repair in these approaches appear to be much greater than the complete in vivo approach previously reported.

2. The previously mentioned methods to measure DNA repair will be used to assess the effect that one type of adduct (e.g., BPDEI-DNA) has on the repair of another type of adduct (e.g., methylated baser). To our knowledge, there have been no studies which explored this type of interaction between carcinogens. This is a relevant environmental toxicology problem since we are exposed simultaneously to a variety of DNA damaging agents.

3. The major nitrosamines detected in tobacco and tobacco smoke include 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) which are formed from nicotine. Carcinogenicity data on NNK and NNN support the possible role of these nitrosamines in tobacco related carcinogenesis. NNN was found to induce nasal cavity tumors in F-344 rats when given by subcutaneous injection, while administration of this nitrosamine in drinking water gave primarily esophageal tumors. NNK was observed to be a more powerful carcinogen than NNN in the rat, including a high incidence of lung and liver tumors as well as nasal cavity tumors. The metabolism of these nitrosamines has been studied in in vitro systems. There have been no reported studies on DNA adduct formation by metabolites of these compounds. Since a large number of people are continuously exposed to NNN and NNK, in vivo studies on the DNA damage induced by these chemicals and on the repair of such damage in both target and non-target tissues/cells is appropriate.

4. The relationship between initial amounts of damage to DNA produced by an initiating agent (e.g., BP) and the subsequent development of neoplasia by a promoter has been examined in the two-stage mouse skin model. A study by Slaga and coworkers suggest that the initial amount of BPDEI-DNA forms correlated with tumor yield whereas the persistence of this adduct was not important. We will explore the relationship between tumor yield and initial amounts and persistence of adducts in the two-stage liver model system. The determination of these relationships would be helpful in assessing the mechanisms of initiation of carcinogenesis.

5. We will continue to explore the mechanisms by which anti-oxidants and AHH inducers inhibit BP-induced neoplasia. The role of induction (or repression) of specific isozymes of P-450 by these anti-carcinogenic agents in the inhibition of BP-induced neoplasia has not been clarified. We will examine this aspect of the problem in detail (in collaboration with Drs. Philpot and Bend). It is hoped that understanding the mechanism of action of these agents might permit the rational design of more potent carcinogenic compounds.

6. The possibility that the transformation of a normal cell into a tumorigenic cell involves the activation and concerted expression of several proto-oncogenes may provide new insights into long-standing problems in chemical

carcinogenesis. Firstly, the tissue (cell) selectivity of certain carcinogens may be reflected in their inability to activate all of the required oncogenes in the resistant tissues. Alternatively, the set of oncogenes activated by the carcinogen may be the same in both susceptible and resistant tissues but the concerted expression leads to neoplasia only in certain tissues. Secondly, the classification of chemicals as initiators, promoters, complete carcinogens, may become clearer as we better understand the sequential requirements for activation of oncogenes in the various animal model systems. Thirdly, low-dose and species-to-species extrapolation of bioassay data may become more reliable from examination of oncogene activation and expression in animal model systems for carcinogenesis. We will examine the activation and expression of oncogenes in various animal model systems. Emphasis will be on tissue selectivity for oncogene activation and on the kinetics of oncogene expression. All of the oncogene events that will ever occur have already occurred in tumor cells. In order to study the possible sequence of oncogene activation and expression, animal model systems must be used. Initially, tumor induced by chemicals being tested in the National Toxicology Program (NTP) will be examined. Tumors induced by furan, furfural and tetranitromethane have already been examined for expression of activated oncogenes. We also have collected numerous spontaneously arising tumors in animals (from NTP bioassay program) and will compare oncogene activtion in these spontaneous tumors with oncogene activation in chemical-induced tumors.

The long-term goal is to investigate the kinetics of oncogene activation and expression. We want to establish the earliest time after carcinogen administration at which the transforming genes can be detected and/or expressed. Much of this work will be done in collaboration with Dr. Stuart Aaronson, National Cancer Institute (NCI), and Dr. Bob Maronpot, NTP.

C. Publications (past 18 months)

Adriaenssens, P.I., White, C.M., and Anderson, M.W.: Dose-response relationships for the binding of benzo(a)pyrene metabolites to DNA and protein in lung, liver and forestomach of control and butylated hydroxyanisole-treated mice. Cancer Res. 43: 3712-3719, 1983.

Hoel, D.G., Kaplan, N.L., and Anderson, M.W.: Implication of nonlinear kinetics on risk estimation in carcinogenesis. Science 219: 1032-1037, 1983.

Adriaenssens, P.I., Sivarajah, K., Boorman, G.A., Eling, T.E., and Anderson, M.W.: Effect of aspirin and indomethacin on the formation of benzo(a)pyrene-induced pulmonary adenomas and DNA adducts in A/HeJ mice. Cancer Res. 43: 4762-4767, 1983.

Anderson, M.W.: Effective biological dose. In Bresnick, E. (Chairman): National Research Council, Committee on Pyrenes and Selected Analogues, Report on Polycyclic Aromatic Hydrocarbons: Evaluation of Sources and Effect. Washington, D.C., National Academy Press, Chapter 5, 1983.

Anderson, M.W. and Bend, J.R.: In vivo metabolism of benzo(a)pyrene (BP): formation and disappearance of BP metabolite-DNA adducts in extrahepatic tissues versus lung. In Rydstrom, J., Montelius, J. and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. New York, Elsevier Science Publishers, 1983, pp. 459-468.

Kulkarni, M. and Anderson, M.W.: Persistence of benzo(a)pyrene metabolite-DNA adducts in lung and liver of mice. Cancer Res. 44: 97-101, 1984.

Kulkarni, M., Angerman-Stewart, J., and Anderson, M.W.: Detection of in vivo DNA repair synthesis in mouse liver and liver induced by treatment with benzo(a)pyrene or 4-nitroquinoline-1-oxide. Cancer Res. 44: 1547-1550, 1984.

Stowers, S.J. and Anderson, M.W.: Ubiquitous binding of benzo(a)pyrene metabolites to DNA and protein in tissues of mouse and rabbit. Chemico-Biol. Interact. (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 70132-05 LP

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Intestinal Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C.M. Schiller	Research Chemist	LP	NIEHS
Other:	C.R. Shoaf	NIH-Postdoctoral Fellow	NRSA	NIEHS
	M.W. King	NIH-Postdoctoral Fellow	NRSA	NIEHS
	D.E. Chapman	Toxicologist	UNC	NIEHS

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LAB/BRANCH
Laboratory of Pharmacology

SECTION
Cell Pharmacology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Our research focuses on the development and use of animal model systems to study th regulation of gastrointestinal functions. Of particular concern are the regulation of intestinal absorption and metabolism of nutrients, and the alteration of these normally occurring events in response to oral exposure to biologically active environmental toxins. Currently, our investigations involve the systematic examination of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) on normal lipid assimilation. The mechanism of the physiologic changes is monitored with a combination of *in vitro* and *in vivo* techniques. In particular, our studies include examination of 1) chylomicra formation, transport and metabolism, 2) mesenteric lymph chylomicra and very low density lipoproteins, 3) dose-related responses to TCDD exposure and 4) genetic component of metabolic response.

PROJECT DESCRIPTION

A. Regulation of Intestinal Metabolism

Nature of problem: There is an increasing awareness of the gastrointestinal tract as a major metabolic organ system, however, the mechanisms of metabolic regulation of its unique processes are not well defined. The absorptive processes are complicated and require specially differentiated epithelial cells. The assimilation of ingested lipid involves the interaction of several organs in order to digest, solubilize, absorb, synthesize and transport the necessary components through the lymph to the organisms. Many ingested environmental toxins are lipophilic and, therefore, would be expected to follow the absorptive route of the dietary lipid which may interfere with normal absorption of lipid nutrients and lipid-soluble vitamins. TCDD is a lipophilic toxin known to affect lipid metabolism in humans after accidental exposure. We have observed effects on intestinal lipid uptake and lipoprotein metabolism. Therefore, TCDD is a useful tool for examining perturbations of lipid assimilation in an animal model. In particular, TCDD is a useful probe to the understanding of chylomicra formation, transport and metabolism, and of mesenteric lymph flow. The relationship between lipid absorption and intestinal epithelial cell protein synthesis which has been examined with direct inhibitors of protein synthesis remains an enigma. Because TCDD augments the flow of ingested lipid through the intestinal mucosa as enlarged lipoproteins without stimulating intestinal epithelial cell protein synthesis or the volume of lymph flow, it offers a unique opportunity to study the regulation of these two phases of epithelial cell function independently.

Objectives in near term:

The primary objective is to define the effects of sublethal levels of TCDD on lipid absorption and lipoprotein metabolism in an appropriate animal model (adult male Fischer rat).

Experimental approach and scientific justification:

The animal model employed involves administration of a single low dose of TCDD in corn oil by gastric intubation one week prior to experimentation and/or sacrifice. Under these circumstances, there are marginal changes in body weight, nominal differences in feed consumption, and marked changes in serum parameters, hepatic lipid metabolism, lipid uptake and lipoprotein metabolism. Since TCDD exposure augments lipid absorption, collection of the mesenteric lymph provides an ample source of lipoproteins (chylomicra and very low density lipoproteins, VLDL) of intestinal origin for examination and further experimentation. A convenient method for analyzing the apoprotein content is to radiolabel the apoproteins *in situ* with leucine. To study lipoprotein lipid metabolism, the chylomicra are radiolabeled with palmitic acid.

Recent accomplishments/significance:

Comparison of rats/TCDD sensitivity

Considerable effort was spent in performing detailed 30-day LD50 studies with four (sub)strains of rats so that we could select an appropriate alternate supplier for Fischer (F/344N) rats. The results are that the Charles River/ Fischer (CR/F) rats were most sensitive to TCDD (LD50 = 164 µg/kg, 104-217, 95% confidence limits), the Frederick/Fishcer (F/F) and Charles River/CD (CR/CD) rats were moderately sensitive to TCDD (LD50 = 303, 250-360, and 297, 240-360 µg/kg, respectively) and the currently supplied rats, Harlan/Fischer (H/F), were most resistent to TCDD (LD50 = 340, 281-409 µg/kg). There was no dose response relationship for feed consumption or body weight loss for all doses (0 to 375 µg/kg) in any of the (sub)strains. The range of mean time to death and percent body weight loss prior to death is 25 to 28 days and 37 to 43%, respectively. These results document genetic drift in substrains of in-bred rats. The F/F rats are closest to the H/F rats, which we had used, in LD50 values, however, because the F/F rats are not routinely available at 10-11 weeks of age, we switched to CR/F rats. Many of our key observations obtained with H/F rats were repeated in the CR/F rats at a necessarily lower dose of TCDD, 45 or 60 µg/kg, for e.g., changes in serum paramters and mesenteric lymph composition.

Changes in lipid metabolism in rat

Examination of the mesenteric lymph collected from control and TCDD-exposed rats after administration of a dose of corn oil revealed the following changes in the TCDD-exposed rat: 1) The total lipid absorbed and protein released into the lymph was greater after TCDD exposure; 2) the ratio (by weight) of total triglyceride to total protein obtained during 12 hr of collection of lymph (after administration of corn oil) increased after TCDD exposure; 3) the size of the lymph lipoproteins were greater after TCDD exposure; and 4) the apoprotein composition of the lymph chylomicra and very low density lipoproteins were changed after TCDD exposure, i.e., apo A-I decreased and apo E increased. The clearance of chylomicra triglyceride, isolated from TCDD-exposed rat donors and reinjected into TCDD-exposed rat recipients, was significantly decreased. Several approaches are being employed currently to determine the basis for the decreased clearance rate, e.g., activity of serum lipoprotein lipase with artificial and natural substrates, and removal of chylomicra remnants by the liver (binding and uptake).

Contribution of nutrients to TCDD toxicity in mice:

An alternate model for studying the effects of TCDD on lipid metabolism was the genetically well-defined mouse model, i.e., C57-TCDD-sensitive, DBA-TCDD-resistant and B6D intermediate. The oral 30-day LD50 values for C57, B6D and DBA mice were obtained respectively 182, 296 and 2570 µg/kg. Log dose versus percent mortality response were parallel in all three strains of mice suggesting a common mechanism of lethality. The acute toxicity of TCDD was characterized by dose-related changes in 1) carbohydrate and lipid metabolism and 2) body weight loss in both C57 and DBA mice. One week after exposure to a single oral dose of TCDD, C57 and DBA mice exhibited decreased serum glucose, triglyceride and

cholesterol, and increased liver triglyceride. C57 mice were approximately 10-fold more sensitive to TCDD-induced alterations in these parameters than DBA mice. The effects of fasting (24 to 96 hr) and TCDD exposure on glucose and lipid parameters were compared; significant differences in the responses of these parameters to fasting and TCDD exposure suggest that TCDD-induced changes in metabolism are not entirely the result of starvation. Food consumption was measured in C57 mice and was not affected by TCDD until the animals were moribund, confirming that weight loss and changes in metabolism are not dependent on decreased food consumption.

B. Plans for subsequent year

Clarification of apparent anomalies:

After approximately three years of intensive examination of the effects of TCDD exposure on nutrient absorption and metabolism, several apparent anomalies have emerged. Our initial premise was that because enlarged lipid droplets are observed in the intestinal epithelium after administration of a dose of corn oil to a TCDD-exposed rat there is a significant physiological impairment in lipid metabolism. This in situ observation does not distinguish between blocked lipid absorption and subsequent accumulation of lipid droplets within the intestinal epithelium, and augmented lipid uptake from the lumen and subsequent overloading of the chylomicra formation process. Because high doses of TCDD are associated with significant body weight loss and death, it was logical to conclude that low doses of TCDD block lipid absorption which contributes to the observed wasting. A series of whole animal studies demonstrated that administered corn oil is recovered to an increased amount (2- to 3-fold increases) in the lymph and in the serum after low doses of TCDD exposure. In contrast, in vitro techniques, which afford measurement of specific obligatory steps in the lipid absorptive process, indicate similar values, e.g., uptake of micellar lipids by gut sacs and triglyceride synthetase activity in the intestinal epithelium. Thus, experiments will be designed to examine particular steps in the absorptive process in an attempt to locate the basis for the augmented lipid uptake. For example, a whole animal experiment will involve duodenal infusions of micellar solutions which contain radiolabeled lipid-triglyceride, monoglyceride or fatty acid, and collection of the mesenteric lymph. An alternate approach is to examine the possible structural changes in the brush border and plasma membranes which may affect, respectively, a decrease in unstirred water layer resistance to lipid absorptive (considered by some current researchers to be the rate-limiting step in the lipid absorptive process) and/or an increase responsiveness to a circulating stimuli of the absorptive process (it was recently demonstrated that some lipoproteins isolated from serum bind to the plasma membranes of enterocytes).

In retrospect, another absorptive phenomenal anomaly emerges, namely, that it is possible that while in vitro measurements of amino acid and monosaccharide transport are similar in gut rings from control and TCDD-exposed rats, the rates of transport of these molecules are different in vivo. Thus, a series of in vivo experiments are envisioned which consider this anomaly. Experimental protocols are complicated by possible differences in stomach emptying and by the

inability to cannulate mesenteric lymph ducts in the absence of lipid to "illuminate" them, therefore, serum and/or urine sampling may be necessary.

A curious metabolic anomaly that we recognized early in our work was the decrease in serum glucose and increase in serum triglyceride when the animals are shifting toward body weight loss. Even when rats are fasted for several days this profile is not induced. However, this anomaly may represent the inability of the TCDD-exposed rat to utilize triglyceride. The ad libitum diet is low in fat and yet both serum and liver triglyceride levels are increased after exposure to TCDD. The regulation of circulating triglycerides has received much attention, but there are many unknowns remaining. Since the reinjected chylomicra triglyceride clears at a slower rate when both the donor and recipient are TCDD-exposed rats; it is possible that the serum lipoprotein lipase cannot metabolize the chylomicra and/or the liver cannot recognize/bind/remove the chylomicra remnant. We are continuing our work with serum lipoprotein lipase and various substrates. No one has demonstrated a feedback effect resulting from the inability to clear chylomicra remnants, perhaps for lack of the appropriate probe, however, this remains an intriguing possibility. A liver slice culture system used in conjunction with fluorescent antibody and ferritin-lipoprotein conjugate techniques, and possible ^{125}I-lipoprotein binding studies will be used to monitor serum lipoprotein binding and uptake.

C. Publications (last 18 months)

Alderman, J. A., Walden, R., and Schiller, C. M.: Development of rat liver and colon substrate cycle enzymes in offspring with and without prenatal exposure to a carcinogenic dose of 1,2-dimethylhydrazine. Comp. Biochem. Physiol. 76C: 383-389, 1983.

Schiller, C. M., Shoaf, C. R., and Chapman, D. E.: Alteration of intestinal function by chemical exposure: Animal models. Intestinal Toxicology. Raven Press, 1984.

Schiller, C. M. (Ed.): Intestinal Toxicology, Target Organ Series. Raven Press, 1984.

Schiller, C. M., Walden, R., Chapman, D. E., and Shoaf, C. R.: Metabolic impairment associated with a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin in adult male Fischer rats. Cold Spring Harbor Symposium, Banbury Report (in press).

Three additional TCDD-related manuscripts have cleared in-house and are under consideration for publication.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 70200-10 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Molecular Mechanisms for Regulating the Intracellular Bioavailability of Metals

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Other:	P. Mistry	Visiting Fellow	LP	NIEHS
	P. Goering	NRSA Postdoctoral Fellow	LP	NIEHS

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LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Molecular mechanisms which regulate the intracellular bioavailability of metals such as lead and cadmium have been studied in both mammals and marine invertebrates. High affinity cytosolic lead-binding proteins PbBP of 63,000 (63K) and 11,500 (11.5K) daltons from kidneys of rats have been partially purified by gel and anion exchange chromatography, electrophoresis, and sucrose density gradient analysis. These molecules were found to exhibit dissociation constants (K_d) for lead of 10^{-8}M. Sucrose density gradient analysis (SDGA) of these molecules showed sedimentation coefficients of 2S and 4.6S for the 11.5K and 63K dalton PbBP, respectively. Competitive binding studies on sucrose density gradiantes with cytosol showed displacement of ^{203}Pb by Pb^{II}, Cd^{II} and Zn^{II} ions but not Ca^{II} ions. Cell-free nuclear translocation studies showed both time- and temperature-dependent uptake of ^{203}Pb from kidney cytosol and KCl extraction of these nuclei followed by SDGA indicated the presence of one saturable peak with a sedimentation coefficient of 2S. Cd^{II} and Zn^{II} ions effectively blocked the nuclear uptake of ^{203}Pb. The data indicate that these high affinity PbBP, which act as the initial cytosolic ligands for Pb in the kidney, are capable of mediating the intranuclear translocation of Pb and that this process is competitively blocked by Cd^{II} and Zn^{II}. The 11.5K dalton protein, but not the 63K protein, was also found to regulate the inhibitory effects of Pb on the heme biosynthetic pathway enzyme δ-aminolevulinic acid dehydratase (ALAD). Reversal of Pb-induced inhibition of hepatic ALAD activity was dependent on the concentration of 11.5K dalton PbBP added to the reaction mixture. Kinetic analysis of either hepatic or renal ALAD activity indicated a non-competitive inhibition pattern. Addition of the semi-purified 11.5K dalton PbBP to the assay mixture markedly reduced the inhibitory effects of Pb on the Vmax of the enzyme from either tissue. The data indicate that the 11.5K dalton protein confers partial resistance to Pb inhibition of liver ALAD in vitro and suggests a similar role for this protein in kidney with respect to the resistance of renal ALAD to Pb inhibition.

PROJECT DESCRIPTION

PROJECT 1

A. Biological Roles of High Affinity Cytosolic Proteins in Mediating Cellular Injury from Cadmium and Lead

Nature of problem: The intracellular bioavailability/activity of metals such as cadmium (Cd) and lead (Pb) appears to be regulated to a high degree by binding to specific high affinity cytosolic proteins. These proteins appear to be of two general types: 1) those such as metallothionein (MT) whose synthesis is regulated by divalent metal cations and 2) those such as the recently reported (Oskarsson et al., BBRC 104: 290, 1982) Pb-binding proteins (PbBP) which appear to be normal constituents of cells and which bind metals due to presence of vacant or displaceable binding sites capable of accepting exognous toxic metals with formation of stable bonding arrangements. The net result of this appears to be an effective competition between molecular binding sites for specific metal cations. Those with higher affinities/binding stabilities outcompete those with lower affinities/stabilities. Dependi·g upon a number of factors such as protein turnover, redistribution of metals into "sinks" with greater stability such as Pb intranuclear inclusion bodies or mineral concretions and interactive effects between metals, the more effective protein binding ligands which usually possess multi-dentate SH binding sites will outcompete other intracellular ligands and thereby regulate their intracellular bioavailability and toxic potential. Furthermore, there appears to be competition among high affinity binding ligands for the same metals. Thus, in mammals while most of the intracellular burden of Cd will be bound to MT, there is always a small fraction (non-MT) distributed among proteins such as PbBP which have lower affinties for Cd than MT but far greater affinities than other intracellular ligands. The importance of understanding this phenomenon and further evaluating the capacity of these molecules to bind toxic metals such as Pb and Cd rests with a more complete mechanistic understanding of the nature and extent to which such binding ligands regulate the intracellular bioavailability/activity of these metals, particularly with respect to the nuclear uptake and interaction of these metals with DNA. In addition to explaining the clearly documented interaction between Pb and Cd in kidney on a molecular basis, the presence of these high affinity molecules (PbBP) in the kidney also provides a reasonable explanation for the peculiar insensitivity of the cytosolic enzyme δ-ALA dehydratase to Pb inhibition in the kidney unless other metals which compete for PbBP-binding sites are concomitantly administered.

Hypothesis and specific objectives:

Hypothesis:

The overall hypothesis for this project is that these ligands are target cell-specific and act as the primary determinants of Pb toxicity in these cells by 1) acting as cytosolic receptors which facilitate transport of Pb across nuclear or mitochondrial membranes and/or by 2) acting as cellular storage proteins involved in proximal tubule cell formation of nuclear and/or cytoplasmic inclusion bodies which sequester Pb from sensitive metabolic processes. A dual role

for these components as regulators of nuclear/mitochondrial membrane transport and as cytosolic Pb chelators would provide a unified mechanistic explanation for a number of Pb-specific effects in the kidney which include a marked stimulation of nucleic acid synthesis, formation of nuclear inclusion bodies, and the inhibition of mitochondrial but not cytosolic heme biosynthetic pathway enzymes.

Specific objectives:

1. Characterize the biological roles of PbBP in mediating the nuclear translocation, DNA binding, and effects of Pb in mediating gene expression in the kidney.

2. Elucidate the mechanisms by which PbBP regulate the availability of Pb^{2+} to enzymes of heme biosynthetic pathway in target tissues.

3. Purify and characterize PbBP by amino acid analysis, amino acid sequencing and biophysical techniques to elucidate the nature of the binding site(s) in relation to secondary structure.

Experimental approach:

The biological function(s) of PbBP with respect to the possible role of these molecules as "receptors" is being further investigated by Dr. Mistry *in vitro* using standard cell-free nuclear translocation techniques and DNA cellulose binding. *In vivo* time-course studies using an intravenous Pb-injection model which has been quantitatively characterized by ultrastructural morphometry with respect to formation of cytoplasmic and intranuclear inclusion bodies in renal proximal tubule cells in relation to changes in other organelle compartments will assess movement of ^{203}Pb-labeled PbBP into nuclei at different time points in relation to changes in *de novo* protein synthetic patterns assessed by 2-D gel electrophoresis using ^{35}S protein labeling. In addition, the potential role of PbBP in mediating the unusual Pb effects on the heme biosynthetic pathway enzymes δ-ALA synthetase, δ-ALA dehydratase, and ferrochelatase in kidney will also be assessed by Dr. Goering as part of his NRSA Fellowship. For the above studies partially purified PbBP isolated by gel permeation chromatography will be utilized in order to avoid possible reduction in the biological activity of these molecules usually associated with purified proteins.

Purification of PbBP will proceed by several methods using cytosol preparations (105,000 xg supernatant) from control rat kidneys as the starting material. Initial cytosol fractionation will be conducted by Sephacryl S-200 or Sephadex G-75 gel chromatography followed by DEAE cellulose ion exchange chromatography. Subsequent purification will be conducted by HPLC and/or by affinity chromatography. In addition, gradient gel electrophoresis will be utilized. Protein homology will be assessed by polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, isoelectric focusing, amino acid analysis and amino acid sequencing. Structural characterization of purified PbBP will be conducted by circular dichroism and nanosecond fluorescence studies and ^{1}H NMR in collaboration with Drs. Chignell, Hall and Ribeiro in LMB. Collaborative arrangements for amino acid sequencing and x-ray crystallographic studies will also be undertaken.

Characterization of the metal-binding site(s) for purified PbBP will be conducted by Scatchard analysis using ^{109}Cd and ^{203}Pb and site stability by equilibrium dialysis experiments against EDTA. These data in combination with ^{113}Cd and ^{207}Pb NMR studies and EPR analyses of the site using a PCMB spin probe will be compared to similar evaluations conducted on MT and oyster CdBP with respect to those site characteristics which confer specificity for a particular metal.

Recent accomplishments/significance:

In terms of assessing the biological functions of PbBP cytosolic "receptors", in vitro studies have shown both cell-free nuclear translocation of binding $^{203}PbBP$ and inhibition of ^{203}Pb nuclear translocation by concomitant administration of Cd^{+2}. These effects were observed with kidney but not liver cytosol preparations. Such data hence mean that PbBP meet most of the established criteria for "receptors" with respect to this metal in that they show 1) finite binding capacity, 2) high affinity, 3) ligand specificity, 4) tissue specificity, 5) nuclear translocation and 6) regulation of chromatin binding. The final criterion of correlation with biological response, which is the well-known Pb-induced stimulation of kidney DNA/RNA and protein synthesis is currently under study using the in vivo ultrastructural morphometric model described below.

Ultrastructural morphometric analyses conducted on proximal tubule cells of rats given a single intravenous injection of Pb (3.0 mg Pb/kg) and killed 0, 4, 8, 16, 24, 48, or 72 hours later showed that the volume density of both nuclear and cytoplasmic inclusions in proximal tubule cells increased after 8 hours to reach a maximum at 24 hours. At this point, the volume density of nuclear inclusions rapidly decreased but that of those in the cytoplasm remained on a plateau indicating that the formation of nuclear inclusions using this regimen is a reversible process and that movement of Pb back into the cytoplasm may occur suggesting a nuclear-cytoplasmic exchange process similar to that known for steroid receptors. Studies of Pb-induced effects on renal proximal tubule cell protein synthesis using 2-D gel electrophoresis at 4 and 24 hours after injection have shown the appearance of some new gene products and disappearance of others in the Pb-treated rats relative to controls using ^{35}S methionine/cysteine labeling.

In vitro studies concerning the potential role of PbBP in mediating the unusual effects of Pb on renal heme biosynthesis have shown that kidney ALA-dehydratase (ALAD) activity is about 5- to 10-times more resistant to Pb inhibition than that in liver. Kidney cytosol was incubated with ^{203}Pb followed by Sephadex G-75 or G-150 column chromatography to isolate the 11.5K and 63K PbBP, respectively. Addition of the concentrated 11.5K PbBP to liver cytosol preparations prior to addition of Pb resulted in a 20% increase in ALAD activity relative to assay mixtures without PbBP over a concentration range of 0.1 - 0.4 μM Pb. This effect was not observed with the 63K PbBP or BSA added at equivalent or greater protein concentrations. The data indicate that the 11.5K PbBP confers partial resistance to Pb inhibition of liver ALAD in vitro and suggests a similar role for this protein in kidney.

Results of purification studies using DEAE anion exchange chromatography after Sephacryl S-200 or Sephadex gel chromatography of kidney supernatant showed that both 11,500 and 63,000 PbBP could be resolved into two sharp peaks. PAGE, SDS-PAGE and isoelectric focusing studies of these peaks, however, showed a number of bands. It is presently unclear whether these bands represent a lack of PbBP homogeneity or whether they represent aggregates of homogeneous PbBP. Further purification via HPLC, affinty chromatography, gradient gel electrophoresis and amino acid sequencing, which are now in progress, should resolve this matter and permit biophysical characterization as described above.

The significance of the above findings with respect to PbBP is that these data demonstrate a central role for these target tissue specific ligands in mediating the biological activity of this ubiquitous element, particularly at low dose levels. These molecules hence provide a molecular mechanism for explaining the nuclear translocation of Pb, interaction of Pb with DNA and subsequent genetic and possibly carcinogenic responses associated with this element in the kidney. In addition, these proteins also clearly regulate the availability of Pb to sensitive enzymes (ALAD) in a highly dose-related manner indicating that previously reported tissue differences in ALAD susceptibility to Pb may now be explained on a molecular basis. The importance of this knowledge rests with providing a molecular basis for predicting tissue, age, and sex-related differences to Pb inhibition of sensitive biological indicators such as ALAD.

B. Plans for subsequent year

The research efforts of this laboratory for the next several years are focused on purifying and characterizing PbBP in comparison with other known high affinity metal-binding proteins such as MT or oyster CdBP, such that the relationships between protein amino acid sequence, secondary structure, and formation of the metal-binding sites may be understood. A second area of intense interest concerns further elucidation of the various functions played by these molecules in mediating the intracellular bioavailability/activity of metals such as Cd and Pb with respect to ultrastructural/biochemical manifestations of cell injury.

C. Publications (see Project 2)

D. Abstract

Lead is preferentially toxic to specific cell types in major target organs such as the kidney and brain. Cells of the renal proximal tubule (S_3 segment in particular) show early and marked toxic responses to this element but the factors which influence this predilection of effect are undefined and usually assumed to be related to a simple preferential concentration of metal in these cells from the blood. Intrinsic cell-specific functions or factors (such as reabsorptive transport processes, metabolic pathways or cytosolic binding components) which may potentiate lead accumulation in specific cell types have never been described. Recent studies from this laboratory (Oskarsson et al., BBRC 104: 290-298, 1984) have identified the presence of target tissue-specific cytosolic lead-binding components with molecular masses of 11,500 (11.5K) and 63,000 (63K) daltons in kidney and brain but not in liver or lungs of control rats. Saturation and Scatchard analysis of ^{203}Pb binding in whole kidney cytosol and

in 40% saturated ammonium sulfate precipitated fractions disclosed a class of relatively high affinity sites with an apparent K_d of approximately 50 nM for ^{203}Pb and binding capacity of approximately 41 and 9 pmol/mg protein, respectively. Two ^{203}Pb binding proteins with approximate molecular masses of 63K and 11.5K daltons and a high molecular weight component (>200K) were isolated by Sephacryl S-200 column chromatography. A biphasic increase in total bound ^{203}Pb the 63K and 11.5K protein fractions was observed with an increase in pH. Saturation analysis fo the three isolated proteins disclosed low capacity, high affinity sites with similar apparent K_d values to the cytosol assay. Sucrose density gradient analysis of kidney cytosol demonstrated a three peak profile similar to the Sephacryl S-200 column profile. The sedimentation coefficients of the 11.5K, 63K and the high molecular weight proteins were approximately 2S, 4.6S and 7S, respectively. Competitive binding studies on sucrose density gradients with cytosol showed displacement of ^{203}Pb by Pb^{II}, Cd^{II}, and Zn^{II} ions but not Ca^{II} ions. Cell-free nuclear translocation studies showed both time- and temperature-dependent uptake of ^{203}Pb from kidney cytosol and KCl extraction of these nuclei followed by sucrose density gradient analysis indicated the presence of one saturable peak with a sedimentation coefficient of 2S. The data indicate that these high affinity lead binding proteins which act as the initial cytosolic ligands for Pb in the kidney are capable of mediating the intranuclear translocation and and hence influence the DNA binding of Pb. In addition, renal δ-amino-levulinic acid dehydratase (ALAD) is markedly more resistant to Pb inhibition than hepatic ALAD in vivo. Inhibition of hepatic ALAD activity by Pb was partially reversed by a single concentration of semi-purified 11.5K PbBP in the presence of 0.1 - 0.4 μM Pb, but no protective effect was observed at higher concentrations of Pb. This effect was not observed with the 63K PbBP or BSA added at equivalent or greater protein concentrations, respectively. Reversal of Pb-induced inhibition of hepatic ALAD activity was dependent on the concentration of 11.5K PbBP in the reaction mixture. Kinetic analysis of either hepatic or renal ALAD activity at an IC_{50} concentration of Pb indicated non-competitive inhibition patterns for this enzyme in either tissue. Addition of the semi-purified 11.5K PbBP to the assay mixture markedly reduced the inhibitory effects of Pb on the Vmax of the enzyme in both tissues indicating a metabolic role for the 11.5K PbBP in mediating tissue-specific differences in ALAD sensitivity to Pb inhibition.

PROJECT 2

Evolution of Metal-Binding Proteins and High Affinity Sites

Nature of problem: Numerous studies have shown that soluble low molecular weight metal-binding proteins play an important role in cellular metal metabolism in many species. One particular protein, metallothionein, has been extensively studied in mammalian systems where it appears to play a central role not only in zinc metabolism in normal animals but also in the intracellular binding and biological availability of Cd and Hg in animals exposed to these metals. This protein is characterized by a molecular mass of 6,800 daltons, an unusually high cysteine content (25-30%), an absence of aromatic amino acids and an extremely high metal-binding capacity (7 g atoms metal/mol protein).

Low molecular weight proteins, similar in size to metallothionein, have also been found in a variety of marine organisms. Proteins from cadmium-treated crabs (Scylla serrata, Cancer pagurus) and mussels (Mytilus edulis) have been isolated and classified as metallothioneins on the basis of the above criteria although a number of marked differences from mammalian metallothionein were noted. Other low molecular weight cadmium-binding proteins (CdBPs) from the oyster (Crassostrea virginica) and mussel (Mytilus edulis) are similar in size to metallothionein but do not have the high cysteine and low aromatic amino acid content of metallothionein. Oyster CdBP (molecular weight, 7,400) is characterized by relatively high amounts of aspartate and glutamate, lower cysteine (15%) and a combined total of approximately 6% phenylalanine and tyrosine. Tryptophan is also detectable in the protein by steady-state fluorescence. Furthermore, the metal content of the isolated oyster CdBP is low relative to metallothionein with only 1-3 g atom metal/mol of isolated protein. The relationships between these differences in amino acid composition, metal-binding capacity and structure of CdBP are presently unknown but they are potentially of great significance to understanding the biological function of this protein in vivo and determining its possible evolutionary relationship to metallothioneins in more highly developed species.

Hypothesis:

The hypothesis to be tested is that mammalian metalalothionein may have evolved by a number of convergent mechanisms including: 1) addition of cysteine-based sequences to small proteins (2,000 daltons) such as have been reported in yeasts; 2) substitution of cysteine based sequences into proteins of similar size (oyster CdBP) or 3) gene cleavage with splitting of cysteine-rich sequences from larger proteins (scallop kidney CdBP).

Specific aims:

1. Complete biophysical studies on oyster CdBP which examine the relationship between amino acid sequence, structure and formation of SH-mediated binding sites in relation to other MT-like proteins.

2. Evaluate CdBP's in other species of molluscs, such as the scallop kidney which has a CdBP with an estimated molecular weight of approximately 21,000, to determine if the molecule(s) represent a dimer of a known MT-like protein or if protein chain-shortening has also occurred in the evolution of MT.

Experimental approach:

Purification of CdBP's from oyster or other marine invertebrates and perhaps other non-mammalian species will be conducted following exposure to native Cd^{+2} or ^{113}Cd and these proteins isolated by standard column chromatography methods established in this laboratory. In addition, isolation of proteins by HPLC and gradient gel electrophoresis will also be conducted. Protein homology will be assessed by PAGE, SDS-PAGE, isoelectric focusing, amino acid analyses and amino acid sequencing. Biophysical studies will be conducted on these molecules as described above using circular dichroism, ^{1}H and ^{113}Cd NMR, nanosecond fluorescence and ESR spin probe analysis of the site so that direct comparisons with other known Cd-binding proteins may be made (see sections C and D of Project 1).

Recent accomplishments/significance:

Amino acid sequence studies on oyster CdBP are in progress. The initial results indicate that the N-terminal of CdBP is blocked. Structural analysis of CdBP by circular dichroism showed that CdBP possesses a more ordered structure (50% β-sheet) than MT which is 100% random. Previous studies have shown that the American oyster (Crassostrea virginica) produces a low molecular weight cadmium-binding protein (CdBP) similar in size to metallothionein (MT) but which contains a lower cysteine content than MT and binds only 1-3 g atoms Cd/mole protein versus 7 for MT. Scatchard analysis of ^{109}Cd binding to purified CdBP showed a single class of site(s) with an apparent dissociation constant (K_d) of 10^{-7} M for Cd. Addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to CdBP followed by separation of protein bound and free ionic Cd on small Sephadex G-25 columns demonstrated displacement of Cd from CdBP. Variable SH:Cd ratios from 4.5:1 to 2:1 at saturation were obtained for CdBP by this method in contrast with the 3:1 ratio normally reported for MT. Kinetic analysis of DTNB reactivity with CdBP SH groups, electron spin resonance spectroscopy (ESR) studies using a PCMB spin probe also showed Cd^{+2} displacement from the proteins and that the Cd binding site was present on or near the surface of the molecule. Circular dichroism studies of CdBP incubated in vitro with excess of Cd or Cu disclosed marked reduction in the positive 259 nm Cd-S bond peak but no changes in the UV spectrum. EDTA (1 mM) chelation studies of CdBP showed that like MT release of Cd^{+2} from the protein via this method was an extremely slow process as measured by the observed decrease in the 259 nm circular dichroism peak.

The above data indicate that oyster CdBP shares both similarities and differences with MT. Overall, the characteristics of this protein suggest that one evolutionary pathway for MT involved development of a random coil domain structure from a more ordered protein to permit formation of multidentate sites. Secondly, the evolution of clusters with scalar SH coupling to the bound metal ions increases the efficiency/stability of the site and prevents formation of S-S bonds or random displacement of the bound metal ion (i.e., Cd or Zn) by a metal such as Cu under physiological conditions. Nanosecond fluorescence studies of the tryptophan moeity conducted by Dr. Robert Hall (LMB) showed that the emission spectral peak was "blue-shifted" with a half-life of approximately 2.3 nanoseconds indicating exposure to the buffer.

Given the importance of high affinity metal-binding proteins such as metallothionein in regulating the intracellular biological activity of metals such as Cd^{+2}, it is of interest to consider both the evolutionary patterns of MT-like proteins in nature and the influence of these patterns on protein structure and formation of metal-binding sites. At present, complete data sets (amino acid sequence, secondary structure, ^{113}Cd NMR studies) exist for only a few of these proteins but it appears that convergent evolution involving the insertion of an increased number of SH based sequences and a decrease in PO_4 or COOH binding centers with an increase in random and decrease in β-sheet structure does occur. Another mechanism would involve addition of SH containing sequences to smaller molecules. The obvious net result of these alterations is an increase in both the stability, affinity and specificity of SH-mediated metal binding sites which should increase the effectiveness of these molecules as regulatory proteins for specific metal cations.

The evolutionary patterns of high affinity metal-binding proteins in different species is also of considerable importance since it permits phylogenetic comparisons which greatly aid in elucidating a basic understanding of how these proteins and high affinity sites evolved and what chemical or selective advantages may be derived from different types of sites (i.e., clusters). At present, such correlative data between amino acid sequence, structure and formation of metal-binding sites is available for only a few MT or MT-like proteins in different non-mammalian species. Recent reports indicate that a number of other apparently inducible Cd-binding proteins with many characteristics different from MT also exist. These proteins hence provide unique research opportunities for elucidating the evolutionary mechanisms and patterns of the MT family of proteins.

B. Plans for subsequent year

Research efforts for the next several years are focused on the chemical and biophysical characterization of oyster CdBP and scallop kidney CdBP. Once these data are collected, studies from other laboratories, which are in progress, on other species are published then construction of an evolutionary tree for this important class of proteins may be initiated.

C. Publications (past 18 months)

Fowler, B.A., Kardish, R., and Woods, J.S.: Alteration of hepatic microsomal structure and function by acute indium administration: Ultrastructural morphometric and biochemical studies. Lab. Invest. 48: 471-478, 1983.

Woods, J.S., Fowler, B.A., and Eaton, D.L.: Studies on the mechanisms of thallium-mediated inhibition of hepatic mixed function oxidase activity. Correlation with inhibition of NADPH-cytochrome c (P-450) reductase. Biochem. Pharmacol. 33: 571-576, 1984.

Squibb, K.S., Pritchard, J.B., and Fowler, B.A.: Cadmium metallothionein nephropathy: Ultrastructural/biochemical alterations and intracellular cadmium binding. J. Pharmacol. Exper. Therap. 229: 311-321, 1984.

Victery, W.W., Miller, C.R., and Fowler, B.A.: Lead accumulation by rat renal brush border membrane vesicles. J. Pharmacol. Exp. Therap. (in press).

Goering, P.L. and Fowler, B.A.: Regulation of lead inhibition of δ-aminolevulinic dehydratase by a high affinity renal lead-binding protein. J. Pharmacol. Exp. Therap. (in press).

Fowler, B.A.: The role of ultrastructural techniques in understanding mechanisms of metal-induced nephrotoxicity. American Society for Pharmacology and Experimental Therapeutics Symposium on Metal Toxicity in the Kidney. Fed. Proc. 42: 2957-2964, 1983.

Fowler, B.A.: Ultrastructural and biochemical alterations of cellular organelle systems by pernatal exposure to toxic trace metals. Proc. Internat. Conf. on

Developmental and Reproductive Toxicity of Metals. University of Rochester, Rochester, New York, May, 1982, Plenum Press, pp. 437-451.

Fowler, B.A., Abel, J., Elinder, C.-G., Hapke, H.-J., Kagi, J.H.R., Kleiminger, J., Kojima, Y., Schoot Uiterkamp, A.J.M., Silbergeld, E.K., Silver, S., Summer, K.H., and Williams, R.J.P.: Structure, mechanism and toxicity. In Niragu, J. (Ed.): Changing Metal Cycles and Human Health. Dahlem Workshop Reports, Springer-Verlag, 1984, pp. 391-404.

Squibb, K.S. and Fowler, B.A.: Intracellular metabolism and effects of circulating cadmium metallothionein in the kidney following acute treatment. Environ. Hlth. Perspect. 54: 31-35, 1984.

Petering, D.H., Loftsgaarden, J., Schneider, J., and Fowler, B.A.: The metabolism of cadmium zinc and copper in the rat kidney: The role of metallothionein and other binding sites. Environ. Hlth. Perspect. 54: 73-81, 1984.

Fowler, B.A.: The effects of arsenicals on freshwater and marine organisms. In Fowler, B.A. (Ed.): Biological and Environmental Effects of Arsenic. Elsevier/North Holland Press, 1983, pp. 155-170.

Squibb, K.S. and Fowler, B.A.: Biochemical mechanisms of arsenical toxicity. In Fowler, B.A. (Ed.): Biological and Environmental Effects of Arsenic. Elsevier/North Holland Press, 1983, pp. 233-270.

Fowler, B.A.: Overview of the environmental arsenic problem. In Fowler, B.A. (Ed.): Biological and Environmental Effects of Arsenic. Elsevier/North Holland Press, 1983, pp. 271-277.

D. Abstract

The evolutionary patterns of the metallothionein family of proteins in non-mammalian species is an area of intense interest since these data may illuminate genetic mechanisms for the insertion of cysteine based sequences into this protein and the impact of this process on changes in structure and formation of the SH-mediated binding sites which characterize this protein. The American oyster (Crassostrea virginica) produces a low molecular weight cadmium-binding protein (CdBP) which is similar in size to metallothionein (MT) but which has a lower cysteine content and binds less Cd and no Zn. Studies of the CdBP binding site via kinetic analysis of the titration rate of SH groups by 5,5'-dithiobis-(2-nitrobenzoic) acid showed a single phase reaction versus biphasic from MT. ESR studies using a parachloromercuribenzoate spin probe also showed Cd^{+2} displacement from the protein, and loss of the positive 259 nm circular dichroism peak suggesting that the Cd binding site was near the surface of the molecule. Circular dichroism studies of CdBP incubated in vitro with excess Cd disclosed a 40-50% reduction in the 259 nm Cd-S bond peak but no marked changes in the UV spectrum suggesting geometric alteration of the site. EDTA (1 mM) chelation studies of CdBP showed that, like MT, release of Cd^{+2} from the protein via this method was an extremely slow process which required days at 20°C as measured by the observed decrease in the 259 nm Cd-S circular dichroism peak. The above data indicate that the metal binding site of CdBP is similar to mammalian MT in

that it is SH-mediated and resistant to EDTA chelation of Cd^{+2} but also different with respect to the apparent localization of the site near the surface of the molecule, monophasic response to SH titration and unusual circular dichroic properties after addition of excess Cd^{+2}. These data taken in concert with ongoing amino acid sequence and structural studies of oyster CdBP suggest that one evolutionary pathway for MT may involve the insertion of cysteine based sequences into a more ordered protein with concomitant changes in structure and metal-binding site formation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80001-12 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Microsomal Mixed-Function Oxidase Systems: Specificity and Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, CA

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

For a number of years we have been investigating the cytochrome P-450 monooxygenase systems (P-450 systems) of rabbit liver and lung. The results of these studies have provided explanations for some of the differences that have been noted between the abilities of these two tissues to metabolize xenobiotics. The key to these explanations has been the discovery that the concentrations and types of cytochrome P-450 isozymes present in liver and lung differ significantly. At present we are primarily concerned with the distribution of cytochrome P-450, form 5, in liver and lung and its response to various inducers and repressors. Until recently, the evidence for isozyme 5 in the liver could be obtained only by immunochemical methods. Now, however, this enzyme has been purified from the livers of rabbits treated with phenobarbital. A direct comparison of isozyme 5 from liver and lung can now be carried out. This structural, immunochemical and catalytic comparison should provide information sufficient to establish if these proteins are identical. The second major isozyme of rabbit lung, form 2, is also under investigation. The results of our previous work have not provided any evidence that the liver and lung enzymes are different. However, we now have some evidence that microheterogenous forms of isozyme 2 may exist in the liver but not in the lung. The immunochemical techniques now available make detailed investigations of the P-450 systems of tissues other than liver and lung (the two tissues with the highest concentrations of cytochrome P-450) possible. Because one of the isozymes (form 5) studied in our laboratory is highly active in the metabolism of aromatic amines, we have initiated an investigation of the P-450 system of rabbit bladder, the target tissue for the carcinogenic effects of these compounds. Rabbit bladder containes isozymes 2, 5 and 6 but does not contain isozyme 4. Therefore, the bladder has the enzymes required for the metabolism of aromatic amines to reactive products. Like the lung, however, the bladder has little or no ability to N-hydroxylate 2-acetylaminofluorene and can activate this compound only after is has been deacetylated to 2-aminofluorene.

PROJECT DESCRIPTION

A. Microsomal Mixed-Function Oxidase Systems: Specificity and Function

Nature of problem: Cytochrome P-450 monooxygenase systems (P-450 systems) metabolize a multitude of endogenous and exogenous chemicals. As a result, P-450 systems are involved in a myriad of vital metabolic processes associated with the detoxication and elimination of xenobiotics and the synthesis and catabolism of biochemicals. However, the combination of monooxygenation and broad substrate specificity also produces a number of deleterious effects associated with the formation of carcinogenic, mutagenic and other toxic products from compounds that may have little or no inherent biological activity.

P-450 systems are composed of NADPH cytochrome P-450 reductase, a flavoprotein, and an undetermined number of isozymes of cytochrome P-450. Because these isozymes have distinctly different substrate preferences, the specificities and metabolic capacities of P-450 systems are, for the most part, determined by the types and concentrations of the isozymes present. These factors, type and concentration, vary markedly with respect to species, age, tissue, cell type, and a number of other determinants, and can be significantly altered by treatment of animals with a wide variety of chemicals (inducers/repressors).

Objectives in near term:

Although we have described a number of differences and similarities between the P-450 systems of rabbit liver and lung, recent advances in immunochemical techniques have made it possible to greatly extend these comparisons and to relate them to other tissues. Our present interest centers around the distribution of cytochrome P-450, form 5, an enzyme that is highly active in the metabolism of aromatic amines to mutagenic products. Purified isozyme 5 from liver and lung will be investigted with respect to structure and function in order to determine if microheterogeneity exists for this protein. The substrate specificity of isozyme 5 will be established in purified systems and in microsomal preparations. The role of isozyme 5 in the metabolism of aromatic amines, especially in the rabbit bladder microsomal fraction, will be established. Lastly, attempts will be made to isolate analogous proteins from species other than rabbit.

Experimental design:

The overall design of our experiments includes three basic approaches: manipulation of P-450 systems by treatment of animals with various chemicals; analysis of microsomal activities and enzyme levels; and determination of the specific activities of given P-450 isozymes in microsomal and purified systems. This approach incorporates a number of immunochemical, biochemical and analytical techniques, including "Western blotting", direct immunochemical quantitation, antibody inhibition, protein purification methods (ion-exchange, hydrophobic, affinity, molecular sizing), peptide mapping, amino acid analysis, and an assortment of colorimetric, fluorometric, and radiometric assays. Experiments are designed with the intent of associating changes in the overall substrate specificity of the P-450 systems with specific alterations in the profile of cytochrome P-450 isozymes.

Recent accomplishments:

Cytochrome P-450, form 5, is one of the major isozymes of P-450 found in rabbit lung. We have previously purified this protein and investigated its structural, catalytic and immunochemical properties. On the basis of antibody inhibition studies and analysis by "Western blotting" we have tenatively identified isozyme 5 in rabbit liver where it amounts to only 1-2% of the total P-450 but catalyzes 50 to 70% of the hepatic microsomal metabolism of several aromatic amines to mutagenic products. The hepatic concentration of P-450, form 5, increases about 10-fold following treatment of rabits with phenobarbital. In an effort to determine if the form 5 proteins from liver and lung are identical or are microheterogenous forms of the same enzyme, we have purified form 5 from rabbit liver and lung. Purification from rabbit heptic microsomal preparations was carried out with chromatography on DEAE-Sepharose, DEAE-cellulose, CM-cellulose, HA-agarose, and calcium phosphate gel. Purification has been assessed by gel electrophoresis and "silver staining" of proteins. Contamination of preparations with isozymes 4 and 6 has been evaluated by "Western blotting". The methods used have shown clearly that the generally accepted criteria for determining the purity of P-450 preparations are inadequate.

The rabbit pulmonary microsomal fraction is much more active than the hepatic fraction in the metabolism of 2-acetylaminofluorene (AAF), 2-aminofluorene (AF) and 2-aminoanthracene (AA) to mutagenic products as determined by the Salmonella mutagenesis assay. This tissue difference is a reflection of the relative concentrations of cytochrome P-450, form 5, in lung and liver. The results of collaborative studies with Dr. Bend's laboratory show that AF is the major product formed from AAF by rabbit pulmonary microsomal preparations. No N-OH-AAF (less than 5 pmol/min/mg protein) can be detected by HPLC analysis of extracts from incubations of pulmonary microsomes with AAF. Therefore, the pulmonary metabolism of AAF to mutagenic products is initiated by deacetylation to AF; N-hydroxylation of AAF contributes little, if any, to the pulmonary microsomal activition of AAF. Incubation of AAF with rabbit hepatic microsomal preparations results in the formation of seven-times more AF than N-OH-AAF. Both of these products are further metabolized to mutagenic metabolites via the formation of N-OH-AF and the relative mutagenic activity of N-OH-AAF is twice that of AF over a wide range of concentrations. The differences in the rates of formation and mutagenic activities indicate that the primary (70-80%) route of metabolism of AAF to mutagenic products is initiated by deacetylation. This finding does not coincide with the generally accepted view that N-hydroxylation of AAF is a required and rate-limiting step in the formation of mutagenic products from AAF. The mutagenic activities of AF and AAF metabolized by the microsomal fraction from rabbit liver and lung correlate with the rates of formation of N-OH-AF as determined by HPLC.

The microsomal fraction from rabbit bladder contains unexpectedly high concentrations of cytochrome P-450, form 5, along with forms 2 and 6. Form 6, which is a minor isozyme in rabbit liver and lung, is the major P-450 in bladder. However, the concentration of form 6 in the bladder is not increased following treatment of rabbits with polycyclic aromatic hydrocarbons, a class of compounds known to induce the synthesis of form 6 in rabbit liver and lung.

The relatively high concentration of form 5 in bladder accounts for the finding that the rabbit bladder microsomal fraction is more active than the hepatic microsomal fraction in the N-oxidation of 2-aminofluorene (2-AF). Like the lung, the bladder has little or no ability to N-oxidize 2-acetylaminofluorene (2-AAF) because of the absence of isozyme 4. The microsomal "activation" of 2-AAF is initiated by deacetylation to 2-AF in rabbit bladder.

B. Plans

A number of approaches will be used to further characterize the structural, immunochemical and catalytic properties of cytochrome P-450, form 5. Amino acid compositions, N- and C-terminal sequences and "peptide" maps will be obtained for P-450, form 5, purified from liver and lung. Peptides will be detected by direct (protein staining) and indirect (immunochemical) methods. Similar work will also be carried out with different fraction of P-450, form 2, isolated from liver. This work will provide answers regarding the microheterogeniety of P-450.

The relative contributions of isozymes 4 and 5 in the metabolism of aromatic amines to mutagenic products in liver preparations will be evaluated by antibody inhibition and kinetic studies. Also, the role of deacetylation versus N-oxidation of 2-acetylaminofluorene will be investigated as a function of substrate concentration.

The induction and repression of isozymes 2 and 5 will be investigated. The induction of 2 and 5 by phenobarbital is the only example of the synthesis of apparently unrelated isozymes taking place simultaneously. The use of monoclonal antibodies and cDNA probes will allow us to establish if these isozymes are truly unrelated.

C. Publications (past 18 months)

Robertson, I.C.G., Serabjit-Singh, C.J., Croft, J.E., and Philpot, R.M.: The relationship between increases in the hepatic content of cytochrome P-450, form 5, and in the metabolism of aromatic amines to mutagenic products following treatment of rabbit with phenobarbital. Molec. Pharmacol. 24: 156-162, 1983.

Robertson, I.C.G., Aune, T., Serabjit-Singh, C.J., Croft, J.E., Bend, J.R., and Philpot, R.M.: The role of rabbit cytochrome P-450, form 5, in the pulmonary and hepatic metabolism of aromatic amines to mutagenic products. In Gustafsson, J.-A. and Rydstrom, J. (Eds.): Extrahepatic Drug Metabolism and Clinical Carcinogenesis. Amsterdam, Elsevier/North Holland Biomedical Press, 1983 (in press).

Serabjit-Singh, C.J., Domin, B.A., Bend, J.R., and Philpot, R.M.: Immunochemical and biochemical evidence of the presence of cytochroem P-450 in rabbit heart and aorta. IBID.

Serabjit-Singh, C.J., Albro, P.W., Robertson, I.G.C., and Philpot, R.M.: Interactions between xenobiotics that increase or decrease the levels of cytochrome P-450 isozymes in rabbit liver and lung. J. Biol. Chem. 258: 12827-12834, 1983.

Aune, T., Robertson, I.C.G., Vanderslice, R., Croft, J., Dybing, E., Bend, J., and Philpot, R.: Deacetylation to 2-aminofluorene is the major initial reaction in the microsomal metabolism of 2-acetylaminofluorene to mutagenic products by preparations from rabbit lung and liver. Molec. Pharmacol., 1984 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80002-13 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enzymes Metabolizing Chemicals: Effectors of These Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: James R. Fouts Research Pharmacologist LP NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Cell Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

This project combined with Z01 ES 80003-10 LP and continued as Z01 ES 80039-01 LP

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80003-10 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Xenobiotic-Metabolizing Enzyme Activity in Skin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Dr. James R. Fouts Research PharmacoloP NIEHS

COOPERATING UNITS *(if any)*

Biometry Branch, Histology

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Cell Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project combined with Z01 ES 80002-13 LP and continued as Z01 ES 80039-01 LP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80007-13 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Conjugation and Oxidation Pathways for Xenobiotic Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John R. Bend	Chief	LP	NIEHS
Other:	E. Cheung/J. Horton	Visiting Fellows	LP	NIEHS
	L. Dostal	NIH-Postdoctoral Fellow	LP	NIEHS
	R. Brigelius	Visiting Associate	LP	NIEHS
	D. Brier	Chemist	LP	NIEHS
	C. Harris	Biologist	LP	NIEHS
	C. Serabjit-Singh	Chemist	LP	NIEHS
	O. Hernandez	Chemist	LMB	NIEHS

COOPERATING UNITS *(if any)*

Arrhenius Laboratory of Biochemistry, Stockholm University; Laboratory of Molecular Biophysics, NIEHS; Laboratory of Pulmonary Function and Toxicology, NIEHS; Department of Biochemistry, Vanderbilt University

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	3.4	2.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epoxides are frequently formed as metabolites of unsaturated hydrocarbons by cytochrome P-450-dependent monooxygenase activity. Many arene and alkene oxides are known to react covalently with macromolecules and to transform cells in vitro, suggesting they are ultimate carcinogens, mutagens or cytotoxins. We are studying various aspects of the enzymatic formation and subsequent metabolism of epoxides in relationship to cell-selective and organ-selective toxicity of compounds metabolized to epoxides by both hepatic and extrahepatic tissues. Particular attention is given to the respiratory tract because this is a common site for pollutant-mediated damage. We are currently investigating stereochemical aspects of the P-450-dependent oxidation of styrene to styrene 7,8-oxide in microsomal and reconstituted monooxygenase systems, stereochemical and kinetic aspects of the biotransformation of model alkene (styrene 7,8-oxide) and polycyclic arene (benzo(a)pyrene 4,5-oxide) oxide substrates by cytosolic and purified glutathione transferases of diverse origin, the distribution and characteristics of components of the cytochrome P-450-containing monooxygenase system in vascular endothelium and the status of the tripeptide glutathione, which is important in detoxication of electrophilic metabolics, in perfused lung, Clara cells, alveolar type II cells, alveolar macrophages and tracheal cells (mixed) isolated from rabbit lung before and after exposure to electrophilic metabolites or conditions of oxidant stress (e.g., exposure to paraquat).

PROJECT DESCRIPTION

A. Conjugation and Oxidation Pathways for Xenobiotic Metabolism

Nature of problem: Unsaturated hydrocarbons, such as the polycyclic aromatic hydrocarbons and alkylated benzenes, occur as common environmental pollutants. Potentially toxic electrophilic compounds, including epoxides, are formed as in vivo and in vitro metabolites of hydrocarbons. We are investigating the chemical/biochemical characteristics of the enzymes responsible for formation and subsequent biotransformation of toxic hydrocarbon metabolites in liver and extrahepatic tissues. Attention is focused on the cytochrome P-450-dependent monooxygenase system and the glutathione transferases with regard to expression of target organ and/or cell toxicity.

Objectives in near term:

1. To investigate the stereoselectivity of purified glutathione transferases with selected alkene and arene oxide substrates. Where indicated, because of unique stereochemical properties, selected glutathione transferases will be purified and characterized. (The marked differences we have observed in stereoselectivity of glutathione transferases with the K-region polycyclic arene oxide, benzo(a)pyrene 4,5-oxide suggest that this parameter will be useful for the functional characterization of these dimeric proteins and their subunits. This hypothesis is currently being tested.)

2. To determine the nature and degree of stereoselectivity of the cytochrome P-450-dependent monooxygenase catalyzed conversion of styrene to styrene 7,8-oxide in microsomes and reconstituted systems containing highly purified cytochrome P-450 isozymes. (Styrene is a relatively small aromatic compound that is a substrate for all cytochrome P-450 isozymes we have tested to date. It is also metabolized via a single major pathway, i.e. formation of R- or S-styrene 7,8-oxide. Consequently, we feel that this is a good molecule for comparing the active site topography of different forms of cytochrome P-450. Because the two styrene 7,8-oxide enantiomers vary in mutagenic activity, we are also testing the hypothesis that stereoselective oxidation of styrene will affect the degree of genotoxicity observed.)

3. To identify by immunochemistry and immunocytochemistry the isozymes of cytochrome P-450 present in aorta, heart and smooth muscle of rabbit and to accurately determine the monooxygenase activities associated with these forms of P-450 in these tissues. (The vascular endothelium represents the largest surface area in intimate contact with circulating chemicals. We are investigating the ability of this tissue to oxidatively metabolize exogenous compounds.)

4. To study the glutathione (GSH) status (i.e. biosynthesis, degradation, release) in Clara cells, alveolar type II cells and alveolar macrophages freshly isolated from rabbit lung before and after exposure to chemicals that are known to alter GSH status (e.g. paraquat, styrene 7,8-oxide). (Reaction with GSH is an important detoxication reaction for many chemical toxins. We are investigating relationships between GSH status and xenobiotic-mediated target cell toxicity in lung).

Experimental approach and scientific justification:

General:

An integrated experimental approach is used, and studies are conducted with purified enzymes, subcellular fractions of various tissues, freshly isolated pulmonary cells, isolated perfused organs and intact animals. With this combination of procedures we are generally able to pursue a problem to a logical conclusion. Investigations in systems with intact cellular structure are especially important for those chemicals whose activation/deactivation depend on sequential enzymatic reactions localized in different cellular organelles or in different cells/tissues. Highly purified, radiolabeled substrates are used in most experiments. Metabolite isolation, identification and quantitation are most frequently accomplished using high performance liquid chromatography (HPLC) and scintillation counting. Detailed characterization (^{13}C-NMR, NMR, mass spectroscopy, etc.) and synthesis of metabolites when required, are routinely accomplished in collaboration with scientists in the Laboratory of Molecular Biophysics. The Western blotting technique of Towbin et al. with subsequent immunochemical staining is used both for identification (by monomeric molecular weight) and quantitation (by densitometry) of cytochrome P-450 and glutathione transferase isozymes. Cytochemistry of tissue sections by immunofluorescence and immunoperoxidase staining is used to determine the cellular localization of specific isozymes of cytochrome P-450 or glutathione transferases.

This combination of chemical, biochemical and pharmacological approaches allows us to investigate the chemical and biochemical mechanisms involved in xenobiotic activation and detoxication at the organ, cell and molecular levels.

Recent accomplishments:

1. Stereoselectivity of glutathione transferases (GT): An HPLC system was developed which separates the four diastereomeric glutathione adducts of benzo(a)pyrene 4,5-oxide (BPO). We have shown that cytosolic glutathione transferases of different species or tissues vary markedly in their stereopreference for BPO or styrene 7,8-oxide (SO). For example, rat liver showed a preference for R-configured oxirane carbon atoms with both substrates (R/S = 3.9 for BPO, 2.8 for SO), whereas rabbit liver preferentially attacked S-configured epoxide carbon atoms (R/S = 0.07 for BPO, 0.60 for SO). Both the nature and degree of stereoselectivity varied for GT in cytosol of different rat tissues with BPO. Rat testes (R/S = 5.5), small intestine (4.4) and lung (2.5) preferentially reacted with an R-configured oxirane carbon but kidney (0.21) was selective for S-configured carbons. Collaborative studies with Bengt Mannervik, Stockholm University, have shown the stereoselectivity of three different human GT (placenta π , liver μ, and liver α-Σ) with BPO varies. In concert these data indicate that the stereoselectivity of GT with model epoxides is a useful parameter for their functional characterization, and for studies of the topography of the substrate binding site. We are currently using this approach in our laboratory with GT purified from various sources, including rabbit liver and lung.

2. Stereoselective oxidation of styrene to styrene 7,8-oxide (SO): Experimental conditions were selected so that at least 95% of the metabolically generated SO is enzymatically converted to its diastereomeric GSH derivatives, which are then resolved by HPLC. Rates of styrene monooxygenase activity were greater in rat hepatic microsomes (13.1 ± 4.5 nmol/min/nmol P-450) than in those of rabbit (2.4 ± 1.2). Stereoselectivity also differed in rat and rabbit hepatic microsomes, where S-SO and R-SO are the favored products, respectively (R-SO/S-SO = 0.7 ± 0.1, rat; and 1.2 ± 0.1, rabbit). Treatment with phenobarbital resulted in increased production of R-SO in liver microsomes of both species. However, induction with β-naphthoflavone only affected the rat, where is markedly increased R-SO formation. Rabbit lung also was stereoselective for formation of R-SO (R-SO/S-SO = 1.6 ± 0.2). Reconstituted monooxygenase systems containing those forms of P-450 known to be present in rabbit lung (i.e. isozymes 2, 4 and 6) demonstrated that all isozymes metabolized styrene, and at fairly similar rates (3.5 to 6.5 nmol/min/nmol P-450). These studies were carried out in collaboration with Dick Philpot, NIEHS. However, the stereoselectivity varied; R/S ratios ranged from 0.88 ± 0.01 with isozyme 6 to 2.01 ± 0.05 with isozyme 2. Studies with inhibiting antibodies to P-450 forms 2 and 5 and pulmonary microsomes agreed very nicely with results in the reconstituted system with regard to isozyme stereoselectivity and catalytic activity.

In a recently initiated collaborative study with Fred Guengerich, Vanderbilt University, we have determined the rate of styrene metabolism and its stereoselectivity with P-450 isozymes purified from rat liver. There is one major P-450 isozyme purified from untreated rats (UT_A), PB-induced rats (PB_B) and β-NF-induced rats (βNF_B) that is relatively efficient at catalyzing the oxidation of styrene in reconstituted monooxygenase systems (20, 28 and 12.5 nmol/min/nmol P-450 for UT_A, PB_B and βNF_B, respectively). Moreover, the observed stereoselectivity of each purified form of P-450 (R/S = 0.6, 0.7 and 1.25, respectively) reflects the alteration that occurs in hepatic microsomes of untreated (R/S = 0.7) versus PB-induced (0.9) and βNF-induced (1.25) rats. The fact that styrene is metabolized by one major oxidative pathway (i.e. formation of SO) by all P-450 isozymes tested to date suggests that the stereoselectivity of this reaction may serve as a useful functional comparison for P-450 isozymes of diverse origin.

3. Cytochrome P-450 isozymes in aorta and heart of the rabbit: Western blots of heart and aorta microsomal fractions demonstrated the presence of immunoreactive proteins of monomeric molecular weight equivalent to those of P-450 forms 2, 5, and 6 and NADPH-cytochrome P-450 reductase. Treatment with TCDD resulted in the induction of form 6, while levels of the other enzymes were unchanged. Trace amounts of 7-ethoxyresorufin O-deethylation were observed in microsomes from aorta or heart of microsomes from rabbits treated with TCDD. Preliminary data indicata that the content of these proteins in smooth muscle of the aorta may be significant (i.e. immunoreactive monooxygenase elements may not be concentrated in the intima as suggested by cytochemical data). Both immunoreactive proteins and NADPH-cytochrome c reductase activity are detected in microsomes from aortas which have been stripped of endothelium. No benzphetamine demethylation activity (<100 pmol/min/mg protein) was observed, however, consistent with the minimal content of P-450, form 2 indicated by Western blotting.

The vascular endothelium represents the largest surface area in intimate contact with circulating chemicals, both endogenous and exogenous. The contribution of this tissue, if metabolically active, would be important not only because of size but also because of proximity to all other tissues. Clearly, the nature, amount and activity of metabolic activation/detoxication enzymes, such as P-450, must be determined if a correlation between vascular metabolism of chemicals and disease states is to be established. Moreover, delineation of the distribution of these enzymes in functionally diverse tissues may provide insight to their physiological roles.

4. Intracellular glutathione (GSH) status of perfused lung and isolated rabbit lung cells: The effect of paraquat was determined on GSH status in the perfused rabbit lung. Perfusion for 60 min with paraquat (1 mM in perfusion medium) had no effect on reduced GSH or GSH-protein mixed disulfide concentrations in lung but significantly increased oxidized GSH (GSSG) and $NADP^+$ concentrations. When glucose was omitted from the perfusion medium the changes in GSSG and $NADP^+$ levels were accentuated. ESR studies, in collaboration with Ron Mason, NIEHS, demonstrated that the paraquat radical is formed in Clara cells and alveolar type II cells but not in alveolar macrophages isolated from the rabbit. Studies with the antibody to NADPH-cytochrome c reductase indicate that the radicals are being formed intracellularly and not by lysed or damaged cells.

The reduced GSH level in the different lung cell types (>90% viability by trypan blue exclusion test) was demonstrated to be Clara cells, 2.08 ± 0.65 nmol/10^6 cells; type II cells, 0.45 ± 0.12 nmol/10^6 cells; and macrophages 2.93 ± 0.56 nmol/10^6 cells (n = 8 or 9 in each case)). (HPLC analyses conducted by Mike Meredith, Vanderbilt University.) Type II cells demonstrate a time-dependent loss in viability and GSH content when incubated in minimum essential medium (MEM). Under the same conditions Clara cells are able to maintain GSH levels for up to 60 min with only a small decrease in viability; macrophages remain more than 90% viable over a 90 min incubation and have maintained or increased GSH content. Depletion of intracellular GSH can be achieved by exposure to diethylmaleate (DEM) for 20 min in MEM devoid of sulfur amino acids. The concentration of DEM selected (200 μM for Clara and type II cells, 500 μM for macrophages) results in a depletion of GSH to 40% of original content with no additional loss in viability.

GSH, and the GSH transferases, have an important role in the detoxication of reactive intermediates. GSH is also part of an important protective mechanism for oxidant stress. For these reasons we are interested in the relationships between GSH status and chemically-mediated target cell toxicity in the lung, and are studying organ and cell selective aspects of GSH biosynthesis and depletion.

B. Plans for Subsequent Year

1. Stereoselectivity of glutathione transferases (GT): The stereoselectivity of purified GT from rat and man will be investigated in detail using selected polycyclic arene oxides as substrates. GT will be purified from rabbit liver and lung because cytosol from these sources shows a marked stereoselectivity for S-configured oxirane carbons. In these studies particular attention will be given to subunit composition (i.e. homodimers versus heterodimers) and

to the hypothesis that stereoselectivity with arene oxides will be a useful parameter for the functional classification of GT at the subunit level. Immunochemical comparisons of GT of diverse origin will also be accomplished using the Western blotting procedure.

2. Stereoselective oxidation of styrene to styrene in 7,8-oxide (SO): Studies of the stereoselective oxidation of styrene in reconstituted monooxygenase systems containing cytochrome P-450 isozymes purified from rat liver by Dr. F. Guengerich, Vanderbilt University, will be completed. In addition, the stereoselectivity of this reaction will be determined in liver microsomes from several humans. Finally, mechanistic studies will be conducted on the oxidation of styrene by peroxidative systems, such as by co-oxidation by prostaglandin H-synthase. (This part of the work will be in collaboration with Dr. Tom Eling, NIEHS.) Styrene is a widely used monomer in the plastics industry and is found as a contaminant in cigarette smoke and drinking water. These factors, in concert with the knowledge that SO is genotoxic, demonstrate that detailed knowledge of the metabolic pathways for styrene biotransformation and of relationships between styrene metabolism and its toxicity are desirable.

3. Cytochrome P-450 isozymes in aorta and heart of the rabbit: Future work will include the use of sensitive radiochemical assays that can be used with and without isozyme P-450-specific inhibiting antibodies to determine the catalytic activities in vascular tissue and to distinguish the relative activities in endothelial lining cells versus underlying smooth muscle. We hope to extend enzymatic and immunochemical analysis to freshly isolated and/or cultured vascular cells and to determine the effects of exogenous modulators on the cytochrome P-450-dependent monooxygenase system in these cells. It seems likely that an understanding of the susceptibility to toxic chemicals and the physiological and metabolic functions of various cell types will result from the determination of cellular contents, enzyme activity and characterization of the xenobiotic-metabolizing enzymes which they contain. We are applying this approach to the endothelium and muscle layer of heart and aorta.

4. Intracellular glutathione status of lung and lung cells: The ability of sulfur amino acids (L-cysteine and L-methionine) to support GSH biosynthesis in Clara cells, alveolar type II cells and alveolar macrophages isolated from rabbit lung under conditions of normal and depleted GSH status will be determined. The effects of lung cell exposure to paraquat and styrene 7,8-oxide on intracellular levels of GSH, GSSG and protein-GSH mixed disulfides will also be evaluated. The stereoselectivity of model alkene and arene oxide metabolism by glutathione transferases of pulmonary cells will also be studied. We intend to extend these studies to cultured tracheal and pulmonary cells for comparison to observations made with freshly isolated cells. The respiratory tract is an important site for toxicity mediated by chemicals converted to reactive intermediates, and by oxidant stress. GSH participates in important detoxication pathways for both types of insult, and consequently, studies of its status in lung are of importance.

C. Publications (past 18 months)

Foureman, G.L., White, N.B., and Bend, J.R.: Biochemical evidence that winter flounder (Pseudopleuronectes americanus) have induced hepatic cytochrome P-450-dependent monooxygenase activities. Can. J. Fish. Aquatic Sci. 40: 854-865, 1983.

Jones, K.G., Holland, J.F., Foureman, G.L., Bend, J.R., and Fouts, J.R.: Induction of xenobiotic metabolism in Clara cells and alveolar type II cells isolated from rat lungs. J. Pharmacol. Exp. Ther. 225: 316-319, 1983.

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Anderson, M.W. and Bend, J.R.: In vivo metabolism of benzo(a)pyrene: Formation and disappearance of BP metabolite-DNA adducts in extrahepatic tissues versus liver. In Rydstrom, J., Montelius, T., and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogens. New York, Elsevier, 1983, pp. 459-467.

Robertson, I.G.C., Aune, T., Serabjit-Singh, C.J., Croft, J.E., Bend, J.R., and Philpot, R.M.: The role of cytochrome P-450, form 5, in the pulmonary and hepatic metabolism of aromatic amines to mutagenic products. In Rydstrom, J., Montelius, T., and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogens. New York, Elsevier, 1983, pp. 25-26.

Serabjit-Singh, C.J., Domin, B.A., Bend, J.R., and Philpot, R.M.: Immunochemical and biochemical evidence of the presence of cytochrome P-450 monooxygenase components in rabbit heart and aorta. In Rydstrom, J., Montelius, T., and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogens. New York, Elsevier, 1983, pp. 253-254.

Lee, I.P., Suzuki, K., Nagayama, J., Mukhtar, H., and Bend, J.R.: Hormonal regulation of cytochrome P-450-dependent monooxygenase activity and benzo(a)-pyrene metabolism in rat testes. In Rydstrom, J., Montelius, T., and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogens. New York, Elsevier, 1983, pp. 351-362.

Foureman, G.L. and Bend, J.R.: The capabilities of fish and other aquatic organisms for xenobiotic metabolism. In Proc. Int. Congress of Pesticide Chemistry, 5th, Tokyo, 1983, pp. 3363-3368.

Foureman, G.L. and Bend, J.R.: The hepatic glutathione transferases of the male little skate, Raja erinacea. Chem.-Biol. Interactions 49: 89-104, 1984.

Little, P.J., James, M.O., Pritchard, J.B., and Bend, J.R.: Benzo(a)pyrene metabolism in hepatic microsomes from feral and 3-methylcholanthrene-treated southern flounder, Paralichthys lethostigma. J. Environ. Pathol. Toxicol. Oncol. 5: 309-320, 1984.

Bend, J.R. and Serabjit-Singh, C.J.: Xenobiotic metabolism by extrahepatic tissues: Relationship to target organ and cell toxicity. In Mitchell, J.R. and Horning, M.G. (Eds.): Drug Metabolism and Drug Toxicity. New York, Raven Press, 1984, pp. 99-136.

Yagen, B., Ben-Zvi, Z., Foureman, G., Hernandez, O., Ryan, A.J., Cox, R.H., and Bend, J.R.: The metabolism and excretion of glutathione conjugates of styrene oxide administered to the winter flounder, Pseudopleuronectes americanus, a marine teleost: Identification of the corresponding S-cysteine derivatives as major urinary metabolites. Drug. Metab. Disp. (in press).

Aune, T., Robertson, I.C.G., Vanderslice, R.R., Croft, J.E., Dybing, E., Bend, J.R., and Philpot, R.M.: Deacetylation to 2-aminofluorene is the major initial reaction in the microsomal metabolism of 2-acetylaminofluorene to mutagenic products by preparations from rabbit liver and lung. Mol. Pharmacol. (in press).

Little, P.J., James, M.O., Foureman, G.L., Weatherby, R.P., and Bend, J.R.: 1-^{14}C-Hexadecane disposition in the spiny lobster, Panulirus argus and the American lobster, Homarus americanus. J. Environ. Pathol. Toxicol. Oncol. (in press).

Bend, J.R., Foureman, G.L., Ben-Zvi, Z., and Albro, P.W.: Biochemical responsiveness of fish to certain chemical carcinogens: Possible association with variability of hepatic monooxygenase activities in feral winter flounder (Pseudopleuronectes americanus) and relevance to testing for carcinogenesis and monitoring for selected environmental toxins. Symposium on The Use of Small Fish Species in Carcinogenicity Testing. Monographs Nat. Cancer Inst. (in press).

Pritchard, J.B. and Bend, J.R.: Mechanisms controlling the renal excretion of xenobiotics in fish: Effects of chemical structure. Drug Metabolism Reviews (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 80038-01 LP

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Suicide Inhibitors of Cytochrome P-450: Isozyme and Tissue/Cell Selectivity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pharmacology

SECTION
Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Microsomal monooxygenase systems contain multiple isozymes of cytochrome P-450 which contribute differentially to the oxidative metabolism of endogenous and exogenous substrates; isozyme differences in K_m, V_{max}, regioselectivity and stereoselectivity are common. Hence, modulation of the relative amounts of various P-450 isozymes can have pronounced effects on chemical metabolism and toxicity. For this reason we are studying isozyme selectivity and tissue/cell selectivity of suicide inhibitors of cytochrome P-450. The suicide inhibition by 1-aminobenzotriazole (ABT) and some of its novel N-alkylated derivatives, which we synthesized and characterized, is being studied in rabbit lung and liver. Although ABT is a potent suicide inhibitor, it shows little P-450 isozyme selectivity. N-benzyl-ABT, on the other hand, is both potent and highly selective (but still not specific; it destroys isozymes 2 and 6 associated enzyme activity but does not significantly affect isozyme 5 catalyzed activity). Synthesis of N,N-disubstituted-ABT derivatives is in progress and these compounds, once available, may demonstrate enhanced isozyme selectivity. In a related project, the chemical nature of the alkylbenzene metabolites, which selectively destroy pulmonary (versus hepatic) cytochrome P-450, and the biochemical nature (e.g., isozyme specificity, involvement of other enzymes and cofactors) of the pathways involved are being elucidated. Results to date demonstrate at least two distinct pathways for metabolic activation, one relying only on the presence of NADPH for suicide destruction, the second relying both on the presence of NADPH and alcohol dehydrogenase.

PROJECT DESCRIPTION

A. Suicide Inhibitors of Cytochrome P-450: Isozyme and Tissue/Cell Selectivity

Nature of problem: Microsomal monooxygenase systems generally contain multiple forms of cytochrome P-450, each of which may contribute to the overall oxidative metabolism of a substrate in a different manner; isozyme differences in regioselectivity, stereoselectivity, K_m and V_{max} are common. Consequently, modulation of the relative amounts of P-450 isozymes can have pronounced effects on chemical metabolism, and on toxicity. The administration of different enzyme inducers to animals results in the selective induction of certain P-450 isozymes and the repression of others. Suicide inhibition of cytochrome P-450 offers another potential method for affecting this monooxygenase system in an isozyme and tissue selective manner. Such inhibition, if isozyme specific, will allow the assessment of the catalytic functions of the remaining P-450 isozymes for both endogenous and exogenous substrates. This experimental approach should be equally valid in systems with intact cellular structure and in microsomal preparations.

Objectives in near term (hypothesis tested):

1. To determine the isozyme and tissue/cell selectivity of the suicide inhibition of cytochrome P-450 mediated by the metabolism of 1-aminobenzotriazole and some of its N-substituted derivatives, and to utilize such isozyme selective inhibition to study xenobiotic metabolism in systems with intact cellular structure. (Specific, non-toxic suicide inhibitors offer the potential for protection of humans against specific chemicals to which they are exposed, or by co-administration, for enhanced toxicity of pesticides. In any event this experimental approach will be useful for delineating physiological functions of cytochrome P-450 at the isozyme level, our primary interest).

2. To delineate the chemical nature of the reactive metabolite(s) of alkylbenzenes, such as p-xylene, which selectively destroy pulmonary versus hepatic cytochrome P-450, and to determine which P-450 isozymes are responsible for this suicide catalysis (alkylated benzenes are common chemicals of commerce and chemical/biochemical aspects of their metabolic activation/detoxication pathways require thorough investigation).

Experimental approach and scientific justification:

An integrated experimental approach is used and studies are conducted with purified P-450 isozymes, subcellular fractions of various tissues, freshly isolated or cultured cells, isolated perfused organs and with intact animals. Required novel inhibitors and metabolites are synthesized in the laboratory, and they are characterized by NMR and mass spectroscopy. Metabolite isolation, identification and quantitation are most frequently accomplished by high performance liquid chromatography and scintillation counting or by gas chromatography. Cytochrome P-450 loss is determined by spectral measurement and inhibition of catalytic activity is determined by the use of isozyme selective or specific substrates (e.g. benzphetamine N-demethylation for isozyme 2, N-hydroxylation of

2-aminofluorene for isozyme 5 and 7-ethoxyresorufin deethylation for isozyme 6 in rabbit lung).

This experimental approach allows us to investigate the chemical and biochemical bases of P-450 isozyme selective suicide inhibition.

Recent accomplishments/significance:

Aminobenzotriazole (ABT) was shown to destroy cytochrome P-450 in the perfused lung in a time- and concentration-dependent manner. Up to 90% of total P-450 was inactivated with 10 mM ABT for 60 min. More than 50% of the loss occurred in the first 2 min of perfusion. P-450, form 5 was more sensitive to ABT than forms 2 or 6 but all forms were destroyed by this compound. Thus, ABT is a potent suicide inhibitor of pulmonary P-450 in a system with intact cellular structure or in microsomal preparations but it lacks isozyme selectivity/ specificity.

N-Methyl-, N-butyl- and N-benzyl-1-aminobenzotriozole were synthesized and proof of structure provided by NMR and electron impact mass spectrometry. Exact mass spectrometry was also used to characterize N-benzyl-ABT. N-Benzyl-ABT proved to be a very effective suicide inhibitor of P-450, form 2, as more than 90% of the benzphetamine N-demethylase activity is inhibited in pulmonary microsomes but only 40 ± 5% of the total P-450 is destroyed. However, N-benzyl-ABT also destroys approximately 85% of form 6 catalyzed 7-ethoxyresorufin deethylase activity. N-Butyl-ABT is less potent as a suicide substrate of pulmonary P-450 than N-benzyl-ABT, but seems to share its isozyme selective characteristics. These data demonstrated that isozyme selectivity can be introduced into the ABT molecule by N-substitution. Such substitution does not diminish the potency of these substrates as suicide inhibitors for the sensitive isozymes.

Each of the isomeric trimethylbenzenes was shown to destroy cytochrome P-450 in the isolated perfused rabbit lung, and in pulmonary microsomes in the presence of NADPH. Thus, the mechanism of suicide inhibition is different than that for p-xylene, which also requires the presence of alcohol dehydrogenase in in vitro incubation mixtures to inactivate cytochrome P-450. These experiments have demonstrated that more than one metabolic pathway is involved in P-450 destruction by alkylbenzene.

B. Plans for subsequent year

The synthesis of N-benzyl-N-methyl-ABT is being attempted. This particular molecule is isosteric with benzphetamine at the position in the molecule where oxidation resulting in N-demethylation occurs. It is anticipated, therefore, that this N,N-disubstituted-ABT derivative will demonstrate increased isozyme selectivity relative to N-benzyl-ABT.

Experiments will be performed with ^{14}C-ABT. Questions to be answered include the rate of uptake of ABT by lung from the perfusion medium, the rate of efflux of ABT from the lung, the amount and nature of metabolite(s) formed from ABT in microsomal and reconstituted monooxygenase systems and the amount of ABT-derived

radioactivity bound tightly (covalently?) to microsomal proteins in the perfused lung and in microsomal incubation mixtures. Depending upon the results obtained, we may investigate the pulmonary and hepatic toxicity of ABT after subacute administration. Similar studies are planned with the most specific/selective ABT derivative synthesized (either N-benzyl-ABT or N-benzyl-N-methyl-ABT). Detailed characterization of suicide inhibitors is necessary before experiments conducted with such compounds can be interpreted in an unambiguous manner. Also, potential toxicity and the mechanisms involved for such chemicals should be thoroughly elucidated.

We plan to determine the chemical mechanisms operant in the suicide destruction of pulmonary cytochrome P-450 by the alkylated benzenes, important constituents of industrial solvents. The metabolism of 1,2,3-trimethylbenzene, an effective destroyer of P-450, will be studied with radiolabeled substrate. Structure-activity relationships for the NADPH-dependent destruction of cytochrome P-450 for the alkybenzene series with 1 to 6 methyl groups will be determined. These experiments are a logical extension of our previous observations.

C. Publications (past 18 months)

Mathews, J.M., Dostal, L.A., and Bend, J.R.: Inactivation of cytochrome P-450 in the isolated perfused rabbit lung by the suicide substrate 1-aminobenzotriazole. Fed. Proc. 43: 655, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 80039-01 LP

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*
Xenobiotic Transformation in Isolated Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, leboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*
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SECTION
Cell Pharmacology

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NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.2	2.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Major cell types of the lung and skin are being isolated ans studied for their metabolisms of xenobiotics and selected lipids (in comparison with liver cells). Special studies are being made with Clara, type II and ciliated cells of the lung and sebaceous and basal cells of the skin. Studies of the effects of the techniques used in isolating the enriched populations of cells from tissues are being made with various antibodies to selected cytochrome P-450 isozymes and Western blotting/microdots for quantifying these isozymes and related peptides in cells at various stages in the isolation procedures. A microspectrophotofluorometer is being used to quantify xenobiotic metabolism in single cells and to study variations in this metabolism among cells of an homogenous population. Variations in enzyme activity in periportal and centrolobular liver cells in the perinatal period are compared with these activities in the adult liver. Isolated skin cells are being used to study the metabolism of prostaglandins and arachidonic acid and the site of accumulation of lipid soluble chemicals like the PCBs.

(Combined with former projects -- Z01 ES 80002-13 LP and 80003-10 LP)

PROJECT DESCRIPTION

A. Xenobiotic Biotransformation in Isolated Cells

Nature of problem: Damage to tissues by chemicals is often localized in one part of the tissue or varies in intensity from one area of the tissue to another. With many chemicals, cellular damage is dependent on the metabolism of the chemical by enzymes in the target cells. Localized or area-specific damage implies that different cells or cells in different parts of the organ may have markedly different chemical metabolizing systems. Our research is directed to analyzing and quantifying chemical metabolisms in different cell types and among cells of one type. We have been studying these chemical metabolizing systems in 3 organs: lung, skin, and liver, and have shown that our basic hypothesis is true in each organ -- different types of cells do have different amounts of chemical metabolizing enzymes. The present work will continue and refine these studies and also correlate the chemical metabolizing activities with specific damages to cells by selected chemicals (whose effects depend on metabolism). Studies of effectors of the type and amount of chemical metabolism in specific cell types and organs are also being made (e.g., animal pretreatments with chemicals/drugs to induce or inhibit metabolism; variations in metabolism in the perinatal period). Our recent studies have mostly concerned metabolizing systems in isolated cells of the lung and skin. Isolated cells may be able to more exactly answer questions about individual cell metabolic capacities and regulations than studies on the whole tissue (as with histochemical methods), and cell-cell interactions may be studied with greater control of variables. Isolated cells do quickly change in enzyme composition (especially with regard to chemical metabolizing systems of certain types), and it is necessary to study such changes and how these may obscure the comparison of results with the in vivo situation. Our studies therefore focus on these problems too: 1) isolating enriched populations of specific cell types and 2) studies of changes in enzymes in isolated cells and comparison with cells in the tissue.

There have been 3 major sub-projects active in the last year: 1) chemical metabolizing systems in Clara and type II cells of the rabbit and rat lung; 2) chemical metabolizing systems in isolated cells of skin of hairless mice and the Zymbal's gland of mice and rats and 3) chemical metabolizing systems in hepatic cells from the perivenous versus centrolobular areas in adult versus newborn rats. A fourth project will be incorporated in the first study -- isolation and characterization of the ciliated cells of the airways of rats and rabbits. In each of these projects the basic hypothesis is the same -- that different types of cells will have different chemical metabolizing abilities (qualitative and quantitative) and that this can be related to damage by chemicals which require metabolism for biological activity. In addition in each project there is concern to test for changes in enzymes as the cells are separated and concentrated in fractions. In each project there are studies of how the systems can be manipulated both in vitro and in vivo to better understand their contributions to toxicity and possibly change this. A major part of each of these studies was the development of methods for isolating the cells and enriching cell types so that comparisons could be made of the enzymes and factors affecting them.

Objectives in near term:

a) The lung cells

1. Characterize the xenobiotic and endogenous substrate metabolizing enzymes of the Clara and alveolar type II cells:
 aa) Determine and quantify cytochrome P-450 isozymes in these cells in the rabbit lung.
 bb) Study steroid and lipid metabolisms (progesterone, glucocorticoids, prostaglandins and arachidonic acid) in these cells by cytochrome P-450s and other enzymes.
 cc) Study effectors of these systems -- inducers and inhibitors -- especially with regard to the balance of toxication/detoxication systems, amounts of specific P-450 isozymes, etc.

2. Isolate, enrich and characterize fractions of other lung cell types:
 aa) Isolate the ciliated cells from upper airways and enrich them in fractions for enzyme characterizations -- especially to allow for distinction between ciliated versus Clara and type II cells.
 bb) Quantify cytochrome P-450 isozymes (and xenobiotic metabolisms dependent on these) in ciliated cells of the rabbit trachea/upper bronchi. If possible, compare these with ciliated cells of lower airways. Compare Clara cells from these two parts of the rabbit airway (upper versus lower) -- metabolically and types/quantity of P-450 isozyme(s).

b) The skin cells

1. Characterize the xenobiotic and endogenous substrate metabolizing enzymes of the different cell layers:
 aa) Determine the type and amount of prostaglandin metabolisms in the cell layers.
 bb) Attempt to quantify P-450 isozymes in different cell layers.
 cc) Attempt to enrich particular cell types of the keratinocytes.

c) The liver cells

1. Complete the projects describing the perinatal development patterns of xenobiotic metabolism in periportal versus centrolobular areas.

2. Use isolated hepatocytes to study xenobiotic metabolism in single cells with the microspectrophotofluorometer:
 aa) To develop methods for quantifying enzyme activity in single cells.
 bb) (With these methods) to study the variability of enzyme activity in single cells and from various parts of the liver lobule -- before and after exposure of animals to inducers/inhibitors of xenobiotic metabolism.

d) Hypotheses to be tested

1. Individual cells of the same type may vary in enzyme activity depending on location in the tissue (e.g., periportal versus centrolobular hepatocytes; Clara cells in upper versus lower airways); and this variation will be present and may even be enhanced by animal treatments (e.g., after inducers/inhibitors) or at different ages (newborn versus adult). These variations can translate into susceptibility to damage by chemicals metabolized to active fragments (e.g., CCl_4 in liver; ipomeanol in lung).

2. Lung cells rich in (lipid) inclusion bodies will metabolize steroids and lipids like prostaglandins, arachidonic acid at much different rates and variety than cells without or with few such bodies (e.g., type II cells versus Clara cells versus ciliated cells). Cells rich in endoplasmic reticulum, especially smooth-surfaced, will have higher P-450-dependent metabolisms (chemicals and lipids) than cells poor in ER (e.g., Clara versus type II and ciliated cells; Clara cells of upper versus lower airways).

3. P-450 isozymes will differ in amount and type from one lung cell type to another. Different cell types may have different stability of the P-450 forms (type II versus Clara cell form 2 of P-450 may be different and this will be tested) or P-450 turnover may be different in different cells.

Experimental approach and scientific justification:

a) The lung cells: Methods for isolating enriched fractions of Clara and type II cells from both rat and rabbit lungs have been published and these will be used in further studies. The enzyme pathways to be studied include those already used (7-ethoxycoumarin (7-EC) de-ethylation, 7-ethoxyresorufin (7-ERF) de-ethylation, coumarin hydroxylation, benzphetamine demethylation as well as those specific for selected isozymes of P-450 (e.g., 2-acetylaminofluorene hydroxylation, benzo(a)pyrene hydroxylations, progesterone and testosterone hydroxylations) or key enzymes in arachidonic acid metabolism or prostaglandin metabolism. Metabolism of most steroids will be followed by TLC methods using radioactive substrates and comparisons with known metabolite standards for quantification. This has worked well for estrogen and progesterone metabolism studies, and will be used when we study the glucocorticoids. If large amounts of unknown metabolites are produced (we seem to get this with progesterone and the Clara cell) we may use HPLC for better separation and preparation of material for identification with GC/Mass spectrometry in collaboration with Chemistry. Prostaglandin and arachidonic acid metabolisms will be followed first in collaboration with Drs. Eling and Henke and using HPLC (this is being done with the lung cells and will be done with the skin cells).

Antibodies to specific P-450 isozymes and to cytochrome P-450 reductase have been used for inhibition and quantification studies (e.g. the microdot/blotting techniques for measuring the two major forms of pulmonary P-450 in isolated cells and fractions of cells). Antibody studies can be used to localize enzymes histochemically or to quantify amounts of isozymes in very small quantities of material. These techniques applied to cells at different stages in the preparation/isolation procedures can help us to detect changes in amount and

nature of P-450 in cells (e.g. proteolysis and possible causes for this during isolation of Clara cells; the protective effects of added anti-proteases on P-450 forms in different cell types). These techniques can also be used to determine differences in cell content of P-450 isozymes after animal treatments (e.g., inducers), or between one cell type and another (Clara cells at different parts of the trachea/lung; Clara versus ciliated or type II cells.

The approaches then will combine measurement of enzyme activity and isozyme content in correlation with histology and electron microscopy of the fractions at the time of measurement. This will allow an assessment of structure and function and changes in both of these among different cells and after different treatments. Only in this way can the various hypotheses be most effectively tested. The methods used and their application are chosen to uncover differences in xenobiotic and endogenous substrate metabolisms in major lung cell types and will be the basis of understanding the role of these metabolisms in chemical damage to the lung as well as possible biochemical and physiological functional differences among lung cells and the relations of these to cell placement, cell structure, and interactions.

The cells chosen for study (type II, Clara, ciliated and macrophages) are all major lung cell types and are postulated to play roles in chemical metabolisms in the lung. The study of lipid metabolisms and the choice of steroids to study is based on the role of these cells in lipid storage/release in the lung (type II cells produce, store and secrete surfactant) and on the response of these cells to various lipids/steroids (glucocorticoids cause proliferation of surfactant/type II cells; prostaglandins affect ciliated cell activity).

b) The skin cells: Methods for separating skin cells into fractions corresponding to layers in the skin have been worked out for the hairless mouse. The pathways studied will include selected xenobiotics and steroids/lipids to pinpoint enzyme differences (e.g., P-450 isozymes). If antibodies to lung P-450s work on skin (P450s) we may be able to quantify forms of P-450 -- otherwise we will use substrate specificity until antibodies are available (various substrates which are specific for P-450 forms, as described for the lung cells, above). The lipid metabolism studies will be done in collaboration with Drs. Henke and Eling, using HPLC methods developed in their lab. The methods for separating light and heavy sebaceous cells have been published -- it remains to characterize the metabolic capacities of these cells more exactly.

The studies to be completed here will further characterize the metabolic capacities of different layers of the skin and point to those layers and possible specific cell types with high versus low chemical metabolisms. The prostaglandin/lipid/steroid metabolisms will probably be different in cells which store lipids (e.g. sebaceous cells) versus those not having such functions (e.g., basal cells). Alterations in lipid storage/metabolism are produced by a number of environmental toxins active on the skin -- e.g., PCBs, dioxins. Specific effects of such toxins on particular cells and on particular types of lipid metabolism can be studied using these isolated cells to more precisely pinpoint sites of action and metabolisms affected.

c) The liver cells: Methods for separating periportal and perivenous (centrolobular) cells have been worked out for the adult rat. Methods to do this for the perinatal period will have to be developed -- possibly some combination of elutriation and density gradients. It would be interesting to compare particular isozyme (e.g. P-450) content in both adult and newborn liver cells and the techniques already used for the lung could be applied here for the antibodies available (LM2, LM4, LM6), using the microdot/blotting techniques developed in Dr. Philpot's and Dr. Bend's labs.

Liver cells have so much more enzyme for most drug metabolisms that they will be used to develop methods useful for assay of enzyme activity in single cells. The methods now used with the fluorescent microspectrophotofluorometer employ substrates like 7-EC or 7-ERF. The problems here are that the products are themselves metabolized (conjugated) within the cell as well as diffusing out to add to the general background. With very active cells (e.g., from induced liver) a short period of high rates of product appearance can be quantified using the fluorescent microscope. This is nevertheless deceiving since even such rates are complex products of the appearance of the fluorescent product (umbelliferone) plus the rate of this product disappearing by conjugation inside the cell as well as diffusion out of the cell/field of measurement. With less active cells -- skin and lung -- these problems of "subtraction" of the fluorescent product make quantification of enzyme activity in single cells problematic. Substrates which give products that are not metabolized further, are fluorescent, and do not diffuse or diffuse slowly from the cell are needed. Possibilities are certain chemicals which generate highly polar products, or even insoluble metabolites (e.g., azo compounds that are reduced by P-450-dependent azo reductase, or ethoxylated fluorescein derivatives/esters). Once methods are available/tested on highly active liver cells they will be used to study variability in enzyme activity among "homogeneous" populations of liver cells as well as between cells from different parts of the liver lobule, and from livers of induced/inhibited animals. If the methods are sensitive enough, they will also be applied to individual lung and skin cells -- first to see how variable the activity is in a "homogeneous" population of cells and then to study the effects of inducers on this variation between individual cells.

The studies with liver cells will finish projects begun with Dr. Klinger and point to differences in enzyme activity variation from one part of the liver lobule to another and describe how this pattern develops as the animal ages. This has important implications for changes in liver damage with age -- both in terms of amount of damage as well as which cells are damaged by chemicals metabolized to active toxins (e.g., bromobenzene) -- as a function of animal age. The studies with individual liver cells will describe much more precisely the variability in chemical metabolisms from one cell type to another as well as within a population of the same cells. Quantification of such variability both before and after chemical treatments of the animal would be most important in correlating degree and homogeneity of cellular damage after a given exposure to toxins. Cell-cell interactions and influences could be more readily studied if individual cell responses could be measured. The chemical metabolizing systems are among the most responsive of hepatic enzymes to injury, effectors, and their measurement in single cells would be a major advance in studies of mechanisms of liver damage, diagnosis of such damage, etc.

Recent accomplishments/significance:

a. Lung cells:

1) Initial studies on xenobiotic metabolisms of Clara and type II cells have been completed. 7-EC de-ethylation (primarily by P-450 form 2) and benzo(a)pyrene metabolisms (several P-450 isozymes) were found in both cell types, whereas coumarin hydroxylation (unknown P-450 isozyme(s)) was detected only in Clara cells. Epoxide hydrolase and dimethylaniline N-oxidase activities (microsomal but not P-450-dependent) were found to be 3-10 times more active in Clara than in type II cells (per mg protein).

2) Studies of endogenous substrate metabolisms by Clara and type II cells have begun. Estrogen metabolism was not detected in whole lung (cell digest) using TLC. Progesterone metabolism does occur in both cells (Clara much greater). A large amount of a non-polar metabolite is produced by Clara cells (much less by type II cells, and none in macrophages). In collaboration with Drs. Eling and Henke, we are studying both prostaglandin (PG) and arachidonic acid metabolisms in Clara and type II cells and macrophages from rabbit lung. Prostaglandin F2α is metabolized to at least 3 products in Clara cells (one of these products is possibly a P-450-mediated metabolite). Type II cells also metabolize PGF2α, but less than Clara cells while macrophages gave no detectable metabolism.

3) In collaboration with Drs. Domin and Philpot we are using the Western blotting/microdot techniques to quantify forms of P-450 and assess evidence for proteolysis (presence of peptides) of P-450s during or as a result of the isolation/enrichment processes used to prepare cells from the lung. Blotting and immunochemical staining can be done on whole cell preparations and on microsomal preparations from whole cells. Cytochrome P-450 form 2 and NADPH cytochrome P-450 reductase seem to be more degraded in isolated/enriched Clara cells than in type II cells or macrophages isolated by the same techniques from the same lungs. Even the best preparations of liver or lung cells show the presence of peptides (evidence for proteolysis??) reacting to antibodies to form 2 of P-450. The amounts of these fragments do vary with preparation treatments, and can be affected by protease inhibitors/speed of preparation of cells, additions of antioxidants, etc. The "best" preparative techniques (the least amount of peptides), the most protein in the largest MW band) seem to involve combinations of protease inhibitors, antioxidants etc., but none of these is able to eliminate the peptide bands from any of the cells tested (liver, lung).

4) Single cell metabolism of 7-EC and 7-ERF can be shown using liver cells, especially from induced animals. However, quantification of rate is very difficult for reasons discussed above (3c -- e.g., conjugation of product, diffusion of product). Rates of metabolism estimated from the initial, linear portion of the rate curves give an approximation of P-450-dependent reactions with highly active cells, and vary by factors of 2 to 4 among a population of apparently "homogenous" liver cells. The same techniques applied to lung cells are not able to be used except with Clara cells from rabbit lung (the linear portion of the rate is too short for all other cells tested). Here too rates have varied by factors of 2 to almost 10 in a population of Clara cells. Better

substrates (less diffusion especially of the products are needed for most of the work to be done with non-hepatic cells and these are being sought in collaboration with Dr. Bend's group (e.g., suicide substrates, ethoxyfluorescein ethyl ester). The substrates being tested should give products that are less diffusible than umbelliferone.

5) Ciliated cells are now being prepared from rabbit tracheas in good yield and high purity (several million cells of greater than 80% purity). The major contaminant is Clara cells (range 5 to 15%) and we hope to minimize these by further steps in the enrichment. Preliminary results suggest that elutriation or density gradient centrifugation in 2 phase polymers will work here.

The significance of all these studies in cells from different organs is the confirmation of our hypothesis that different types of cells will have markedly different xenobiotic and endogenous substrate metabolisms and that these can be studied in cells freshly isolated from the tissue. The work with the blot/microdot methods shows that different types of cells are affected differently by the preparation techniques used, or that there is an inherent lability difference for the enzymes (P-450s) being studied -- either way, another indication of cell differences. The markedly higher metabolic capacities of the Clara cell for most of the pathways studied correlates well with the susceptibility of these cells to several chemicals giving toxic metabolites (e.g., CCl_4, ipomeanol) and with the rich cytoplasmic matrix (lots of endoplasmic reticulum). Variation between individual cells in selected metabolic pathways/activities points to cellular heterogeneity whose source needs further study. This might be a product of isolation (protease damage) or be present in situ. The latter seems possible from uneveness of staining (of cells in tissue sections) with various chemicals that semi-quantitatively measure redox components of the P-450-dependent chain. If this variation is present in situ it needs to be documented better and studied for factors affecting it (like inducers, drug treatments, toxins, etc.) since it may be a part of the variation in site of lung/liver damage and severity thereof after chemical exposure. The fact that all cells (liver or lung) isolated by the classical techniques and with the best and shortest methods still show P-450 peptides is surprising. Either P-450 peptides are normally present in cells (and there are higher amounts of these in highly active cells) or all the cells studied have sustained some (at least minimal) damage in isolation. This latter conclusion is hard to accept since such highly active enzymes can be measured in the best cell preparations (liver and lung). Further study is needed to clarify these results and the usefulness of freshly isolated cells of all tissues in mirroring the in situ/in vivo situations.

b. Skin cells:

1. Initial studies of methods for preparing enriched populations of skin cells and for characterizing some of their xenobiotic and endogenous substrate metabolisms have been completed and accepted for publication. In hairless mice the metabolism of several xenobiotics by skin cells (separated into fractions of increasing size and differentiation) did increase from barely detectable in basal cells to high activity in sebaceous and other well-differentiated cells.

Metabolism was increased by treating these mice with inducers (β-napahthoflavone). Sebaceous cells were separated into two classes (heavy and light) with markedly different xenobiotic metabolizing activities. Conjugation reactions by skin cells were measured by both glucuronide and sulfate pathways. The Zymbal's gland in both rats and mice was shown to have significant xenobiotic metabolism (increased by treatment with inducers (β-naphthoflavone)). Although metabolism of progesterone or estradiol could not be detected in mixed cell populations, some initial studies on prostaglandin metabolism may be followed up in collaboration with Dr. Eling's group.

The significance of these studies in skin cells relates both to the basic hypotheses [cells of different types/in different locations in an organ have different metabolisms of xenobiotics/lipids; richness of cytoplasm (especially endoplasmic reticulum) correlates with xenobiotic metabolism activity] and the effects of chemicals on skin cells (differs with layers). The methods developed for separating various cells (in approximation to their position in the skin) could be used in many studies -- to study where and how chemicals accumulate in skin, and the localization of cell damage in skin layers as related to metabolism, etc. The response of skin cells to inducers allows a study of the effects of manipulating these systems on cell damage etc. by chemicals which contact the skin (most environmental exposures).

c. Liver cells:

1. The studies on perinatal development of intralobular distribution of xenobiotic metabolisms were done in collaboration with Dr. Klinger who continues these studies in Jena. This work showed that the distribution of several enzymes of both endogenous and xenobiotic metabolisms pathways were distributed unevenly in adult rat liver lobule (e.g., 7-EC was higher in centrolobular than periportal areas). Separation of periportal from centrolobular cells was attempted with the elutriator and appeared to be possible. When these studies were done with perinatal rats (1 to 3 weeks old) the adult pattern of intralobular distribution was not seen until later ages. This difference in enzyme distribution also correlated with the toxicity of chemicals believed to be metabolized to toxins (e.g., bromobenzene).

2. Studies on P-450s (content and peptides) in isolated liver cells have been described above (A4a3 -- in collaboration with Drs. Domin and Philpot) -- a comparison with isolation techniques used on lung cells and assessment of damage to cells versus enzyme activities and P-450 contents/peptide appearance. Microsomes from liver homogenates prepared as quickly as possible have little detectable peptides reacting with the P-450 antibodies. Yet the best cells, prepared as quickly as possible and by the standard techniques used in most labs have shown peptide bands. These bands increase in number and amount with cell "aging" (shaking at 37°) or perfusion of the liver (with saline to remove blood) before cells are freed by digestion. Storage of cells after isolation (storage in freezer or in liquid nitrogen) does not lead to increased peptides. Exposure of fresh microsomes to proteases used in digesting liver or lung gives increases in peptides at first and then decreases with time (peptides are digested to fragments without antibody reactivity?).

3. Liver cells from animals treated with xenobiotic metabolism inducers can be studied with the microspectrophotofluorometer (can assay metabolism by single cells). This has been discussed above (A4a4).

The significance of these studies with liver cells lies both in comparisons with the results in other tissues and further confirmation of the basic hypotheses that cells in different parts of an organ and of different types will have different xenobiotic and endogenous substrate metabolisms. Liver is a standard to which metabolism studies in other tissues are most often compared. The finding of P-450 "degradation" in the best preparations of isolated hepatocytes makes the findings of this in lung cells less an indication of bad technique than of need to continue investigation as to its meaning and possible relevance to the in vivo situation. It is commonly accepted that freshly isolated hepatocytes are good indicators of the in vivo situation, especially with respect to the metabolism of xenobiotics etc. that we have followed in skin and lung cells. Highly active cells with lots of P-450 may have such high turnovers of P-450 that "peptides" will always be present normally. This needs more study as do attempts to change the amount of peptides in all kinds of cells by gentler treatments, use of protease inhibitors etc.

The finding of no perinatal differentiation of metabolisms in the liver lobule can be correlated with changed liver toxicity in perinatal versus adult animals exposed to chemicals metabolized to toxins (e.g., bromobenzene). This can be studied further -- e.g., do all cells "turn on" at the same time?, is response to inducers uniform in different areas in adult versus newborn?, etc. -- using cell populations isolated by the methods developed or with the microspectrophotofluorometer (with induced cells at least). Documentation of both intercellular (between individual cells) and intralobular differences in xenobiotic/endogenous substrate metabolisms would have great import in understanding liver damage/area and cell specificity of damage and its change with age.

B. Plans for subsequent year:

1. Lung cells:

a. Continue studies on enzyme activities and amounts in Clara and type II cells from rabbit (and rat) lungs. These studies will include progesterone metabolism and identification of the large amount of non-polar metabolite produced in Clara cells. Collaboration with chemistry in isolating and identifying this unknown will be required. Benzo(a)pyrene metabolites in Clara versus type II cells will be studied in induced animals and if possible with cells from control animals (this project suspended last year when all animals appeared to be induced). Prostaglandin (rat) and arachidonic acid metabolisms (rabbit and rat) will be followed in Clara and type II cells in collaboration with Drs. Eling and Henke. We are especially interested in the production of leukotrienes by these two cell types.

b. Continue studies of enzyme changes in isolated cells. In collaboration with Drs. Domin and Philpot, and using the Western blotting and microdot techniques, we will quantify the amounts of P450 forms (starting with form 2) in cells at different stages of the isolation procedures, and compare this with

enzyme activity (especially 7-EC metabolism which appears to be very sensitive and to parallel losses in P-450 amounts) and appearance of peptide bands. We will also use a variety of protease inhibitors and anti-oxidants to give the best "protection" of P-450 by activity and blotting measurements. After determining the "best" procedures we will re-run some key experiments to see how the results differ in terms of enzyme distributions in Clara versus type II cells. This will be compared with liver cells isolated by "standard" versus "best" techniques and the same xenobiotic metabolisms assayed.

c. In collaboration with Drs. Mathews and Bend, we will study the metabolism of certain suicide substrates by isolated lung and liver cells. This use of suicide inhibitors/substrates may allow studies of the correlation of metabolism with cell damage by added chemicals (comparisons with induced cells can also be made to increase the range of changes for correlation of activity/toxicity). This study may also enable us to develop methods of assaying enzymes in single cells since suicide substrates will not diffuse out of the cell and might allow tagging of particular forms of P-450 (if the substrate can be made fluorescent or becomes fluorescent/changes fluorescence on binding).

d. After validation with liver cells, apply methods developed there to the assay of enzymes in single lung cells. This can then be used to follow enzyme activity changes during isolation of cells (at different stages, after different treatments). It may also be possible to study the effects of one cell type on the metabolism of other cells with these methods (adding Clara cells to type II cells for example).

e. Continue study of the isolation and enrichment of different lung cell types. This will begin with ciliated cells and studies of the metabolism of xenobiotics and endogenous substrates (especially prostaglandins, but also drugs that affect ciliated cell functions, and substrates specific in metabolism by particular P-450 isozymes -- e.g., 7-EC). In collaboration with Dr. Coughtrie the metabolism of chemicals via glucuronidations will be assayed so as to differentiate transferases. This will be done in rat cells since rabbit lung is relatively poor in glucuronyl transferases. Ciliated cells will be compared with other cells in both rats and rabbits across a variety of endogenous and xenobiotic pathways. Since ciliated cells expend so much energy in activity of cilia, it would be interesting to study the relation of xenobiotic metabolism to changes in energy production in these cells. Various poisons of cilia, changes in intermediary metabolism and measurements of NADPH/NADP ratios can be studied in isolated cell populations and correlated with effects on xenobiotic metabolisms. Comparisons with liver cells should also be done (a la Thurman). In collaboration with Dr. Bend's group, attempts will be made to quantify P-450 isozymes in ciliated cells (Dr. Ellen Cheung is also working with mixed tracheal cell populations). Ciliated cells from upper versus lower airways will be compared (as will Clara cells) to see how these may differ in enzyme activities, amounts (P-450 forms etc.), morphology etc.

Other lung cell types may be studied in terms of methods to prepare enriched fractions and preliminary characterizations of enzymes etc. The cells of major interest are endothelial cells and type I cells (major proportions of lung cells).

Taken together these studies will substantially add to our knowledge about which cells contribute which kind and how much to the overall metabolism of both xenobiotics and selected endogenous substrates by the lung. Information about several factors which influence or can be used to change lung cell metabolisms will be provided and insight into how changes in metabolism affect lung cell damage by chemicals can be gained. Differences in the regulation of metabolism in different locations can also be determined by the use of these isolated cell preparations. Freshly isolated cells are much preferred to cells grown in culture because of the many findings showing the rapid loss/change in xenobiotic metabolizing systems in all cultured cells as compared with the intact tissues/freshly isolated cells.

2. Skin cells:

a. Collaborative studies on lipid metabolisms by isolated skin cells will continue with Dr. Eling's group. This work will concern the metabolism of prostaglandins and arachidonic acid. The skin is a lipid synthesizing and storing organ and sebaceous cells are especially rich in the P-450 systems which are also involved in lipid metabolisms. Much skin disease is related to changes in lipid metabolism, and many toxins are stored in skin lipids (e.g., PCBs). Lack of scientific staff will preclude other than these collaborative studies for the next year at least.

b. When staff is available, attempts will be made to better separate specific types of cells in the different layers of the skin and to study the actions of chemicals on these cells as well as the variations in xenobiotic metabolism/lipid metabolism from one cell to another. If the microdot/Western blotting methods can be used on skin cells, we will quantify the P-450 isozymes in different cell types and look at the changes in P-450 as the isolation process goes on (are skin cells mor or less susceptible than liver or lung cells to proteases used in isolation; do peptides of P-450 accumulate in skin cells too?). Attempts will also be made to study the distribution of chemicals in various skin cells after treatment of the animals with these chemicals at different doses. First studies will concentrate on the chemicals known to accumulate in skin -- e.g., the PCBs. What cells accumulate/store these chemicals and how does this affect some of the functions of these cells (metabolism of xenobiotics/lipids)? The hypothesis is that chemicals accumulate quite differently in different layers and that lipid-rich cells will accumulate PCBs much more than other cells (e.g., sebaceous cells especially). Cells accumulating PCBs will have markedly different xenobiotic metabolisms/lipid metabolisms too.

3. Liver cells:

a. Collaborative studies with Dr. Klinger will continue -- to study the development of the perinatal liver systems for xenobiotic metabolisms in different parts of the liver lobule. These studies will probably be completed only over a 2 year period or so. Here at NIEHS we will concentrate on methods for separating the periportal and perivenous (centrolobular) cells by elutriation and density gradients, and assaying individual cells for enzyme activity in both the adult and perinatal periods. This will give data about the uniformity/generality of increases in enzyme activity as well as whether the different

areas behave similarly in terms of how cells acquire these enzyme activities. The hypothesis is that the different areas of the liver lobule (portal versus central) turn on at different rates and, that within each area, cells acquire enzymes at different times (individual variability). Effects of enzyme inducers on these processes would be studied since it is already known from histochemical investigations that inducers affect periportal and centrolobular areas differently. Our approach would allow a time course study of this and quantification of the enzyme increases seen.

b. A major use of liver cells will be in developing the microspectrophotofluorometer methods for measuring enzyme activities in single cells -- this technique when available will allow many studies to be made not only with liver (above) but also hopefully with cells from other tissues (lung and possibly skin). Only histochemical techniques are now available for such studies and these are only semi-quantitative, and do not allow studies of enzyme kinetics etc. Attempts will be made to find a substrate which is metabolized to a product that is relatively confined within the cell and which can be detected by the photometer (fluorescent spectra must be different for metabolite versus substrate). Perhaps a suicide substrate would be useful here and collaborative studies with Dr. Bend's group will test a number of these. Substrates which are metabolized to much more polar products may be useful too (see discussion above, A3c, A4ac). Use of 7-EC and 7-ERF will continue so as to build up experience as to the variability in liver cell enzyme activity among individual cells and effects of various treatments on this.

c. Further studies on the presence of P-450 peptides in isolated liver cells will be made. Effects of different isolation procedures on the presence and amounts of these peptides will be studied. Is is possible to eliminate these or are they perhaps "normal"? If the latter, how can we explain the lack of these in microsomes from fresh liver homogenates? These studies will be made in collaboration with Dr. Philpot's group.

As different P-450 isozymes are purified and antibodies made it may be possible to test the stability of different P-450s in cells. These may differ from one location to another, with age and chemical treatments (inducers/inhibitors) and from one tissue to another. Studies will begin here with liver.

C. Publications (past 18 months)

Jones, K.G., Holland, J.F., and Fouts, J.R.: Benzo(a)pyrene hydroxylase activity in enriched populations of Clara cells and alveolar type II cells from control and β-naphthoflavone-pretreated rats. Cancer Research 42: 4658-4663, 1982.

Fouts, J.R.: The metabolism of xenobiotics by isolated pulmonary and skin cells. In Lamble, J.W. (Ed.): Drug Metabolism and Disposition. Current Reviews in Biomedicine 3. Amsterdam, Elsevier Biomedical Press, 1983, pp. 17-21.

Sivarajah, K., Jones, K.G., Fouts, J.R., Devereux, T., Shirley, J.E., and Eling, T.E.: Prostaglandin synthetase and cytochrome P-450-dependent metabolism of (±)benzo(a)pyrene 7,8-dihydrodiol by enriched populations of rat Clara cells and alveolar type II cells. Cancer Research 43: 2632-2636, 1983.

Jones, K.G., Holland, J.F., Foureman, G.L., Bend, J.R., and Fouts, J.R.: Xenobiotic metabolism in Clara cells and alveolar type II cells isolated from lungs of rats treated with β-naphthoflavone. The Journal of Pharmacology and Experimental Therapeutics 225: 316-319, 1983.

Coomes, M.W., Norling, A.H., Pohl, R.J., Muller, D., and Fouts, J.R.: Foreign compound metabolism by isolated skin cells from the hairless mouse. Journal of Pharmacology and Experimental Therapeutics 225: 770-777, 1983.

Pohl, R.J. and Fouts, J.R.: Cytochrome P-450-dependent xenobiotic metabolizing activity in Zymbal's gland, a specialized sebaceous gland of rodents. Cancer Research 43: 3660-3662, 1983.

Pohl, R.J., Serabjit-Singh, C.J., Slaughter, S.R., Albro, P.W., Fouts, J.R., and Philpot, R.M.: Hepatic microsomal NADPH-cytochrome P-450 reductase from little skate, Raja erinacea. Comparison of thermolability and other molecular properties with a mammalian enzyme. Chemico-Biological Interactions 45: 283-294, 1983.

Smith, B.R., Van Anda, J., Fouts, J.R., and Bend, J.R.: Estimation of the styrene 7,8-oxide-detoxifying potential of epoxide hydrolase in glutathione-depleted, perfused rat livers. J. Pharmacol. Exp. Therap. 227: 491-498, 1983.

Pohl, R.J., Coomes, M.W., Sparks, R.W., and Fouts, J.R.: 7-Ethoxycoumarin O-deethylation activity in viable basal and differentiated keratinocytes isolated from the skin of the hairless mouse. Drug Metab. Dispos. 12: 25-34, 1984.

Devereux, T.D.: Alveolar type II and Clara cells. Isolation and xenobiotic metabolism. Environmental Health Perspectives, 1984 (in press).

Coomes, M.W., Sparks, R.W., and Fouts, J.R.: Oxidation of 7-ethoxycoumarin and conjugation of umbelliferone by intact, viable epidermal cells from the hairless mouse. Journal of Investigative Dermatology, 1984 (in press).

D. Abstract

Studies will continue of the variation (qualitative and quantitative) in enzyme activity and composition of the different major cell types of the lung and skin (with comparisons with liver). Emphasis at present is on specific enzyme differences (e.g., P-450 isozymes, lipid and steroid metabolisms) of Clara and type II cells of the rabbit lung. Other major lung cell types will be studied as we are able to develop methods for their isolation (e.g., we are now working on ciliated cells, but will also make attempts to isolate endothelial and type I cells). Once these methods are worked out, studies of selected xenobiotic and endogenous substrate metabolisms by the cells will be made: 1) to see if their postulated cellular functions are reflected in enzyme composition (e.g.,

prostaglandin metabolism and endothelial cells); 2) whether enzyme composition can be related to cellular damage by chemicals (high xenobiotic metabolism and damage by chemicals metabolized to toxins); 3) to assess the role of various cell types in overall lung metabolism of chemicals/lipids -- especially to see if metabolism by non-Clara cells can contribute significantly to overall lung metabolisms. Another major study will assess changes in enzymes resulting from methods used to isolate and enrich cells of particular types. This will use various antibodies to P-450 isozymes and Western blotting techniques/microdots to quantify P-450 isozymes and peptides in cells of various types and at different stages of preparation. These studies will use lung, skin, and liver cells and various measures of enzyme activity as well.

The microspectrophotofluorometer will be used to quantify enzyme activity in single cells and study variations in this activity among cells of a homogeneous population as well as changes effected by animal treatment with chemicals (inducers, inhibitors), different ages of animals (perinatal studies) etc. These studies will begin with liver cells, but be applied to skin and lung cells when better substrates for these studies are found (presently use 7-EC or 7-ERF). Variations in enzyme activity in periportal versus centrolobular liver cells in the perinatal period will be studied with enriched populations (elutriation/density gradient) as well as in single cells.

Isolated skin cells will be used primarily in studies of lipid metabolism -- prostaglandins/arachidonic acid, sites of accumulation of lipid-soluble chemicals (PCBs), and metabolism of xenobiotics by light and heavy sebaceous cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 80040-01 LP

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Pharmacogenetics of Liver Microsomal Cytochrome P-450s

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Negishi	Visiting Scientist	LP	NIEHS
Others:	Y. Kato	Visiting Associate	LP	NIEHS
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	B. Burkhart	Biologist	LP	NIEHS
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COOPERATING UNITS *(if any)*

City of Hope, California

LAB/BRANCH

Laboratory of Pharmacology

SECTION

Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Liver microsomal steroid hydroxylation activities, which are catalyzed by specific forms of cytochrome P-450s, are known to exhibit sex-dependent expression. In inbred mice, the expression of steroid hydroxylations differs from one to another strain. It has been known that 16α-hydroxylation activity of female mice is recessively regulated by a single gene between 129/J and C56BL/6J. This genetic regulation, however, between 129/J and BACB/CJ was found to be autosomal dominant, indicating that more than one allele are involved in this regulation. The sex-dependent expression of hydroxylases in adulthood are irreversibly predetermined by neonatal androgens. It is reasonable to assume that these differences of microsomal cytochrome P-450s in sex may effect sex-dependent drug toxicity, including chemical carcinogenesis. To understand the biological phenomena surrounding sex-dependent and developmental regulation of gene expression in steroid hydroxylases, we have previously purified female predominant mouse testosterone 15a-hydroxylase ($P\text{-}450_{15\alpha}$). Now male predominant mouse testosterone 16α-hydrocylase has been purified and characterized. A specific antibody against $P\text{-}450_{15\alpha}$ or $P\text{-}450_{16\alpha}$ was raised in rabbits, and they completely inhibited only 16α- or 15α-hydroxylation activity. Liver polysomes bearing $P\text{-}450_{15\alpha}$ or $P\text{-}450_{16\alpha}$ mRNA were precipitated by the antibodies from which the cDNA banks were constructed. By the selective hybridization to ^{32}P-labeled probe synthesized from the immunoenriched mRNAs, the cDNAs encoding $P\text{-}450_{16\alpha}$ has been isolated. By the further characterization and isolation of genomic clones with these cDNA probes, we hope to understand the mechanisms of sex- and age-dependent gene expression of these hydroxylases, and gain insight into the role of cytochrome P-450s in steroid metabolisms and sex differences of chemical toxicity. We have also isolated and characterized cDNAs for drug-induced mouse liver microsomal cytochrome P-450. A comparison between androgen-dependent and xenobiotic-dependent cytochrome P-450 genes will provide useful and better information to understand mechanisms of gene expression.

PROJECT DESCRIPTION

A. Developmental Pharmacogenetics of Liver Microsomal Cytochrome P-450s

Nature of problem: Multiple forms of cytochrome P-450 are known to exist and they have overlapping substrate specificities. Some P-450s also exhibit a marked difference in their stereo- and regioselectivity with both endogenous and exogenous compounds. These multiple forms of P-450 can therefore metabolize an unlimited number of chemical compounds. Often, P-450-catalyzed metabolism results in inactivation or detoxication. However, in certain cases the metabolites are more toxic, a process termed metabolic activation. A change of cytochrome P-450 composition may have a pronounced effect on metabolic detoxication versus activation for a given chemical. P-450 isozyme composition is regulated by administration of chemicals such as phenobarbital, polycyclic aromatic hydrocarbons and clofibrate: individual forms of P-450 may be either induced or repressed. Age and sex of animal also contribute to P-450 composition, presumably through hormonal control. In order to completely understand the role of cytochrome P-450 in chemical-mediated toxicity, including carcinogenesis, it will be necessary to eludicdte the molecular mechanism(s) for the regulation of cytochrome P-450 gene expression. Recent information, obtained by using cDNA encoding for specific forms of P-450, has demonstrated that the induction process is the result of gene expression, although the molecular mechanism of P-450 gene expression by chemicals or hormones is virtually unknown.

Liver microsomal steroid hydroxylations are catalyzed by forms of cytochrome P-450. Sex-dependent differences of the steroid hydroxylation activities, such as male specific testosterone 16α-, female specific 15α- or 15β-hydroxylation in rats and mice, is believed to be a reflection of sex-dependent expression of a form of cytochrome P-450 specific for these hydroxylation activities. Previously a specific form of cytochrome P-450 has not been purified. As expected from their sex dependency, the hydroxylation activities are maturated developmentally so that the activities are increased sexual maturation. The age- and sex-dependent expression of hydroxylation activities are irreversibly induced by neonatal androgens (known as "neonatal imprinting"). The molecular mechanisms of this irreversible gene fixation of liver microsomal hydroxylases is an important question to be solved. In inbred mice sex dependenct expression of steroid hydroxylases varies from one strain to another. Testosterone 16α-hydroxylation activity is high in male in 129/J mice, but is equally high in female as well as male C57BL/6 and BALB/c mice. By appropriate crosses between these inbred strains, it was found that sex-dependent 16α-hydroxylation activity is regulated by a single gene. Therefore, the naturally occurring genetic variations in inbred mice would provide a suitable experimental system to investigate the molecular mechanisms of age- and sex-dependent gene expression of steroid hydroxylases.

Objectives in near term:

We have initially asked whether the testosterone 16α- or 15α-hydroxylation activity is catalyzed by a single form of cytochrome P-450 highly specific and active for the hydroxylation or results from an aggregated sum of multiple forms of cytochrome P-450 in microsomes. After we have identified the cytochrome

P-450s involved in overall testosterone hydroxylations, our research will move onto molecular biology by isolating cDNAs and genomic clones encoding the appropriate cytochrome P-450s. Mice are chosen as experimental animals to utilize information from mouse genetics.

1. To establish a mouse genetic model for testosterone 16α- and 15α-hydroxylation activities. The genetics of these activities will be studied utilizing known activity variants in inbred strains (129/J, BACB/CJ, C57BL/6J).

2. To purify the P-450 isozymes specific for testosterone 16α- or 15-hydroxylation. Based on the specific activities, the P-450s will be purified from untreated male and female 129/J mice. Substrate specificity, including stereo- and regioselectivity, of the purified P-450s will be studied with various steroids and with selected exogenous compounds.

3. To produce specific antibodies to the purified forms of P-450 and to purify them by antigen affinity chromatography. The antibodies will be used for immunochemical characterization, quantitation and localization of the P-450 isozymes.

4. To isolate cDNA and genomic DNA clones encoding for the purified P-450 isozymes. By the use of specific antibodies and expression vectors, cDNAs of the P-450s will be isolated and sequenced, and the cDNAs will be used to screen genomic DNA clones from a DNA library. The structures of the isolated genomic DNAs will be determined.

5. To isolate and characterize cDNA and genomic DNA clones encoding for isosafrole induced isozymes of cytochrome P-450.

Experimental approach:

1. Purification of cytochrome P-450 isozymes: The strategy used to purify the P-450 isozymes responsible for testosterone hydroxylation is to follow the specific enzyme activity in eluates from column chromatography using different packing materials. These include octylamino-Sepharose 4B, isobutyl-Sepharose 4B, DEAE Bio-Gel A, CM-52 and hydroxyapatite.

2. Characterization of the purified P-450 isozymes: Substrate specificity, including regioselectivity and stereospecificity, will be studied in reconstituted monooxygenase systems with various steroids, drugs and xenobiotics. The primary amino acid sequences of the purified P-450 isozymes will be studied in collaboration with Dr. J.E Shively, City of Hope, California.

3. Production of specific antibodies to the purified P-450s, and their utilization: Rabbit antibodies to the purified P-450s will be produced and purified by antigen affinity chromatography. With the specific antibodies, immunochemical characterization and quantitation of the P-450s and their translatable mRNAs will be determined during normal development and after treatment with hormones or drugs.

4. Isolation of cDNAs and genomic DNAs of the purified P-450s: Specific polysomes bearing the relevant P-450 mRNAs will be immunoprecipitated from total liver polysomes of male and femal 129/J mice. From the purified poly(A^+)-enriched RNAs, a cDNA library will be constructed with the expression vector, pUC-9. The cDNA encoding for the P-450s will be selected by immunochemical and/or double colony hybridization to the enriched versus non-enriched cDNAs methods. After characterization the cDNAs will be used to isolate genomic DNAs from DNA libraries constructed from male or female 129/J, C57BL/6J, and BALB/CJ mouse genomic DNAs with Charon as the cloning vector.

5. Isolation of cDNAs of isosafrole induced P-450 isozymes: The cDNA library is constructed from sucrose density gradient centrifugation fractionated poly(A^+)-enriched RNAs from isosafrole-treated Ah nonresponsive DBA/2 mice. The G-C tailing method and pBR322 are used to construct the library. The cDNA library will be selected with double colony hybridization to the induced versus non-induced cDNAs and characterized by in vitro translation assays and DNA sequencing. The genomic DNAs will be isolated from the DNA library using cDNAs as hybridization probes.

Recent accomplishments/significance:

1) Purification of mouse liver microsomal testosterone 16α-hydroxylase ($P\text{-}450_{16\alpha}$) and 15α-hydroxylase ($P\text{-}450_{15\alpha}$). The P-450 isozymes specific for testosterone $P\text{-}450_{15\alpha}$ and $P\text{-}450_{16\alpha}$ have been purified. By octylamin Sepharose 4B, hydroxyapatite, DEAE Bio-Gel A, and CM-52 column chromatography steps, the $P\text{-}450_{15\alpha}$ was purified from liver of untreated female 129/J mice to electrophoretic homogeneity as shown by SDS polyacrylamide gel electrophoresis (molecular weight 48,000). The P-450 content of the purified $P\text{-}450_{15\alpha}$ fraction was 14.5 nmol/mg protein, and the peak of the reduced CO-combined spectrum appeared at 451 nm. The specific 15α-hydroxylase activity was 94 nmol/min/nmol P-450 and 1349 nmol/min/mg protein. Purified $P\text{-}450_{15\alpha}$ exhibited stereo- and regiospecificity for testosterone hydroxylation; the only metabolite formed by the purified $P\text{-}450_{15\alpha}$ was 15α-hydroxytestosterone. The purified $P\text{-}450_{15\alpha}$ fraction was also able to catalyze benzphetamine N-demethylation, 7-ethoxycoumarin O-deethylation, aniline 4-hydroxylation, and lauric acid 11- and 12-hydroxylations at various rates.

By DEAE Bio-Gel A, hydroxyapatite, and isobutyl Sepharose 4B chromatography steps, testosterone $P\text{-}450_{16\alpha}$ was purified from liver of untreated male 129/J mice to 85 nmol/min/nmol P-450 and 500 nmol/min/mg protein. The apparent molecular weight of the $P\text{-}450_{16\alpha}$ was 49,000 and a protein whose molecular weight was about 25,000, copurified with the $P\text{-}450_{16\alpha}$. The reduced and CO-combined spectrum of $P\text{-}450_{16\alpha}$ has its absorption maximum at 449 nm. More than 80% of the testosterone metabolites formed by purified $P\text{-}450_{16\alpha}$ was 16α-hydroxytestosterone.

Our accomplishments wil be quite significant by the following reasons: 1) The successful purification of $P\text{-}450_{15\alpha}$ and $P\text{-}450_{16\alpha}$ opened up a new and productive research field concerning the role of androgen-dependent microsomal cytochrome P-450s and molecular mechanisms of sex- and age-dependent gene expression of the hydroxylases. 2) The new idea to purify liver microsomal cytochrome P-450

be studied. Since the androgen-dependent regulation of the hydroxylases is most likely the event of gene expression, isolation and characterization of genes encoded the hydroxylases will be a most important step for our project. Following is a list of research plans.

1) Establish a mouse genetic model for regulation of P-450$_{16\alpha}$ and P-450$_{15\alpha}$ hydroxylases. Based on the hydroxylation activities, the amount of hydroxylase proteins measured by the antibodies and the expression of hydroxylase gene measured by cDNA as probe, the variants in 129/J, C57BL/6J and BACB/CJ and their offsprings will be studied for allelic interaction and segregation.

2) N-terminal amino acid sequences of P-450$_{16\alpha}$ and P-450$_{15\alpha}$. The P-450$_{16\alpha}$ and P-450$_{15\alpha}$ will be purified again to obtain amino acid sequence information. Besides immunochemical and genetic information, this information will be necessary to characterize the isolated cDNAs and genomic DNA clones.

3) Induction of the hydroxylases by exogenous compounds such as phenobarbital, methylcholanthrene, BHT, and PCN and by endogenous compounds such as steroid hormones. The induction will be studied with the antibodies and cDNAs as probes. Sex- and age-dependency of these inductions will also be investigated.

4) DNA sequences of isolated cDNAs encoded P-450$_{15\alpha}$ and P-450$_{16\alpha}$ and isosafrole-induced P-450s.

5) Construction of 129/J, C57BL/6J, and BALB/CJ genomic DNA library and isolation of genomic DNA clones for the hydroxylases. With DNA as cloning vector, genomic DNA library will be constructed and screened by the isolated cDNAs as probes. The isolated genomic DNA clones will be sequenced to elucidate gene structures and differences between alleles.

C. Publications (past 18 months)

Nebert, D.W., Negishi, M., and Eisen, H.J.: Genetic differences in enzymes which metabolize drugs, chemical carcinogens and other environmental pollutants. In Tucker, R.E., Young, A.L., and Gray, A.P. (Eds.): Human and Environmental Risks of Chlorinated Dioxins and Related Compounds. New York, Plenum Publishing Corporation, 1983, pp. 441-462.

Nakamura, M., Negishi, M., altieri, M., Chen, Y.-T., Ikeda, T., and Nebert, D.W.: Structure of the mouse cytochrome P_1-450 genomic gene. Eur. J. Biochem. 134: 19-25, 1983.

Ikeda, T., altieri, M., Nakamura, M., Tukey, R.H., Chen, Y.-T., Nebert, D.W., and Negishi, M.: Characterization of P_2-450 (20S) mRNA. Association with the P_1-450 genomic gene and differential response to the inducers of 3-methylcholanthrene and isosafrole. Eur. J. Biochem. 134: 13-18, 1983.

Nebert, D.W., Nakamura, M., Tukey, R.H., and Negishi, M.: Cloning genes that encode enzymes which metabolize drugs and chemical carcinogens. In Biochemical Basis of Chemical Carcinogenesis. New York, Raven Press, 1983 (in press).

isozymes based on specific enzymatic activity in fractions from columns will be informative for every researchers purifying and characterizing cytochrome P-450.

2) Preparation of specific antibody against purified P-450$_{15\alpha}$ or P-450$_{16\alpha}$. The antibody was raised in rabbits and purified by antigen-Sepharose 4B. Anti-P-450$_{15\alpha}$ or P-450$_{16\alpha}$ antibody was absolute specific for inhibition formation of either 15α- or 16α-hydroxytestosterone by liver microsomes. More than 90% of these hydroxylation activities in microsomes were inhibited by the antibodies indicating that the purified P-450$_{15\alpha}$ or P-450$_{16\alpha}$ is the only major cytochrome P-450 isozyme catalyzing testosterone 15α- or 16α-hydroxylation in untreated female or male mouse liver microsomes. Using the Ouchterlony test, P-450$_{15\alpha}$ and P-450$_{16\alpha}$ did not immunochemically cross-react.

3) Immunoenrichment of polysomes bearing P-450$_{16\alpha}$ or P-450$_{15\alpha}$. With the use of purified anti-P-450$_{16\alpha}$ or P-450$_{15\alpha}$ antibody, liver polysomes bearing P-450$_{16\alpha}$ or P-450$_{15\alpha}$ were precipitated from total liver polysomal fraction. By SDS gel electrophoresis of in vitro translation products, it was shown that mRNA encoding P-450$_{16\alpha}$ or P-450$_{15\alpha}$ was enriched to the degree of 10-20% purity.

4) cDNA cloning of P-450$_{16\alpha}$ and P-450$_{15\alpha}$ mRNAs using pUC-9 as the cloning vector. The cDNA bank was constructed from the immunoenriched mRNA fraction for P-450$_{16\alpha}$ or P-450$_{15\alpha}$. Three-thousand recombinants were screened and ninety-one recombinants were selected for P-450$_{16\alpha}$ clones by hybridization to ^{32}P-labeledf probe synthesized from immunoenriched mRNAs. The size of cDNa inserts for P-450$_{16\alpha}$ ranged from 600 to 16,00 bp. By the same principle, the cDNA bank for P-450$_{15\alpha}$ has been screened to obtain cDNA clone encoding P-450$_{15\alpha}$.

5) Isolation of cDNA and genomic clones for isosafrole-induced mouse liver cytochrome P-450s. Total mRNAs were prepared from isosafrole-treated DBA/2 mouse livers and subfractionated by sucrose density gradient centrifugation. From the mRNAs in 20S regions of fractions, the cDNA library was constructed with pBR322 as the cloning vector. By screening two-thousand recombinants by in situ cloning hybridization with isosafrole-induced versus non-induced liver mRNAs as the probes, pPmi-1 and -2 were selected. pPmi-1 and -2 contain 1,000 and 350 bp cDNA inserts, respectively. Between Ah-responsive and Ah-nonresponsive DBA/2, an induction of polycyclic aromatic hydrocarbons of the 18S and 20S mRNAs were associated with Ah-locus. Genomic clones encoding the mRNAs were isolated from the DNA library. DNA sequences of pPmi-1 and -2 are near completion and characterization of genomic DNA clones has been in progress.

B. Plans for subsequent year

In the past year we have concentrated our efforts on purifying mouse liver microsomal cytochrome P-450s specific for testosterone 16α- or 15α-hydroxylation activity and P-450$_{16\alpha}$ and P-450$_{15\alpha}$ was purified and characterized. The specific antibodies against these hydroxylases was also prepared. Our main objectives will now shift from characterization of the hydroxylase proteins to obtaining cDNA and genomic DNA clones encoding the hydroxylases and allelic variants in the next 1-2 years. With the use of specific antibodies and cDNAs as probes, age- and sex-dependent regulation of the hydroxylases, induction by endogenous and exogenous compounds and intra- and inter-tissue localization will

Nebert, D.W., Chen, Y.-T., Negishi, M., and Tukey, R.H.: Cloning genes that encode drug-metabolizing enzymes. In MacLeod, S.M., Okey, A.B., and Spielberg, S.P. (Eds.): Developmental Pharmacology. New York, Alan R. Liss, Inc., 1983, pp. 51-59.

Harada, N. and Negishi, M.: Mouse liver testosterone 15α-hydroxylase (cytochrome P-450$_{15\alpha}$). Purification, regioselectivity, stereospecificity and sex-dependent expression. J. Biol. Chem. 259: 1265-1271, 1984.

Ohyama, T., Nebert, D.W., and Negishi, M.: Isosafrol-induced cytochrome P$_2$-450 in DBA/2N mouse liver. Characterization and genetic control of induction. J. Biol. Chem. 259: 2675-2682, 1984.

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Harada, N. and Negishi, M.: Mouse liver testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$): Purification, regioselectivity, stereospecificity and immunochemical characterization. J. Biol. Chem., 1984 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 80041-01 LP

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Detection and Quantitation of Cytochrome P-450 Isozymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R.M. Philpot	Research Chemist	LP NIEHS
Others:	B.A. Domin	Staff Fellow	LP NIEHS
	P. Bent	Biological Lab. Technician	LP NIEHS

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Pharmacology

SECTION
Molecular and Comparative Pharmacology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Elucidation of the factors responsible for the substrate specificities of various cytochrome P-450 monooxygenase systems (P-450 systems) requires the means to detect and quantitate the different isozymes of cytochrome P-450. With this information and knowledge of the catalytic activities of the P-450 isozymes, the metabolic capacity of a given P-450 system can, to a great extent, be explained. Techniques for the detection and quantitation of P-450 isozymes have been developed and are being applied to a number of problems. However, these techniques have brought into question the substrate specificities that have been established for some of the enzymes. The reason for this is the sensitivity of the immunochemical methods. In the past, the criterium for the purity of an isozyme preparation used to determine substrate specificity was the appearance of the preparation following electrophoresis and staining for protein. If no impurities were observed, the preparation was judged to be "homogenious". With immunochemical methods we have show that up to 50% contamination with a second P-450 isozyme cannot be detected by protein staining. Such contamination can, however, contribute significantly to the substrate specificity of the preparation. With immunochemical methods we have described a number of major differences between the monooxygenase systems of rabbit liver and lung. The major isozymes of rabbit lung (forms 2 and 5) are minor forms in the liver unless the animals have been treated with phenobarbital. Treatment of rabbits with polycyclic aromatic hydrocarbons results in increases of two isozymes in the liver but only one in the lung. These differences and others are now being investigated in a number of different species including humans. With antibodies to both rabbit and rat isozymes of cytochrome P-450, we have been able to demonstrate that all species examined exhibit similar differences between liver and lung and that all species have analogous isozymes of cytochrome P-450.

PROJECT DESCRIPTION

A. Detection and Quantitation of Cytochrome P-450 Isozymes

Nature of problem: Cytochrome P-450 is a family of isozymes that has been incompletely described. The substrate specificities of these isozymes are determined by examination of the purified proteins in reconstituted systems. Because of this it is imperative that we be able to assess the extent to which purified preparations are contaminated with other P-450 isozymes. At present it is concluded that most isozymes of cytochrome P-450 metabolize a wide variety of substrates but that some isozymes are much more active than others for certain substrates. This conclusion, combined with measurements of individual isozymes in microsomal fractions, results in calculations that divide activities between minor isozymes with high activities and major isozymes with low activities. Our results, in several cases, suggest that as little as 1% contamination of purified preparations with a second isozyme can have significant effects on substrate specificity. Methods for the detection of minor contamination are now available. These methods also make it possible to evaluate the profile of P-450 isozymes in various tissue fractions and to make immunochemical comparisons across species lines.

Objectives in near term:

The ability to detect and quantitate P-450 isozymes of interest is required if they are to be studied in detail. The development of immunochemical methods of high specificity and sensitivity is now underway. These methods are useful for the determination of contamination in purified preparations, detection and quantitation of P-450 isozymes in tissue preparations, and assessment of the extent to which tissues differences and responses to various inducers are similar in different species.

Experimental design:

The primary technique employed for these studies is "Western blotting". This method allows for the immunochemical detection of specific proteins that have been separated on the basis of monomeric molecular weight. With Western blotting it is possible to take advantage of high titer polyclonal antibodies even if they cross-react with proteins other than the primary antigen. It is also advantageous to use antibodies that recognize more than one form of P-450 with Western blotting. For studies that involve more than one species, antibodies to both rat and rabbit isozymes of cytochrome P-450 are used.

Recent accomplishments:

We are now able to detect rabbit isozymes 2, 4, 5, and 6 and rat isozymes b, c, d, e, and f using immunochemical methods. By employing antibodies to the proteins of both species we have shown that a number of species exhibit common properties with respect to the P-450 monooxygenase systems of liver and lung. For example, the major pulmonary isozyme in all species examined is the isozyme found in liver that is induced to the greatest extent by phenobarbital. Also, analogous isozymes are induced in the liver of all species by treatment with

polycyclic aromatic hydrocarbons. Of the two isozymes induced in the liver, only one is found in the lung. The immunochemical similarities between species are such that the same proteins are detected by rat or rabbit antibodies in all cases.

The ability to detect minute quantities of P-450 isozymes has allowed for the measurement of less than 1% contamination of purified preparations by other P-450 isozymes. Contamination at this level cannot be assessed by protein staining, the method commonly used to evaluate purity. The consequences of low levels of contamination can be highly significant. We reported previously that isozyme 5 from rabbit metabolizes the pulmonary carcinogen, benzo(a)pyrene. We can now show that this metabolism is due to contamination of the "5" preparations with small amounts of isozyme 6 which can only be detected by immunochemical methods. This finding has resulted in a complete reevaluation of the metabolism of benzo(a)pyrene by the rabbit lung. In addition, we have found that most of the "purified" isozymes of P-450 available to us (both from our lab and others) have detectable levels of more than one form of P-450.

B. Plans

With the immunochemical methods available we will continue to assess the extent to which the P-450 systems of different species are similar. This work will include an examination of P-450 systems in human tissue samples. Also, we will attempt to establish the substrate specificities of P-450 isozymes in preparations that are free from cross-contamination. Preliminary results suggest that such preparations can be obtained with improved purification procedures.

C. Publications

Domin, B.A., Serabjit-Singh, C.J., and Philpot, R.M.: Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining procedure. Anal. Biochem. 136: 390-396, 1984.

LABORATORY OF PULMONARY FUNCTION AND TOXICOLOGY

THE LABORATORY OF PULMONARY FUNCTION AND TOXICOLOGY
Summary Statement

The Laboratory of Pulmonary Function and Toxicology (LPFT) studies basic biological mechanisms of pulmonary cell biology and biochemistry as well as mechanisms of injury leading to pulmonary disease. The work of LPFT is focused on a few select research topics and is divided accordingly into five groups each having its own central research theme. The groups also collaborate on many projects thus sharing expertise and resources. The five groups are (1) the Cell Biology Group which studies cellular and biochemical mechanisms of differentiation of airway epithelial cells, (2) the Epithelial Carcinogenesis Group which studies mechanisms of neoplastic transformation of epithelial cells from the conducting airways, (3) the Environmental Carcinogenesis Group which investigates the relationships between mutagenesis and carcinogenesis and the molecular basis of multistage neoplastic transformation, (4) the Pulmonary Pathology Group which investigate the cellular and biochemical mechanisms underlying particle and fiber-induced pulmonary fibrosis, and (5) the Biochemical Pathology Group which studies the regulation of secretions in the distal air spaces and its disruption by environmental agents.

Cell Biology Group. One of the long-term research objectives of the Cell Biology Group is to elucidate the regulation of mucin biosynthesis and secretion. Towards this end two important steps were made during the past year: (1) Methods were firmly established to grow, in primary culture, epithelial cells from the tracheas of hamsters and rabbits under conditions which allow expression of the normal differentiated phenotype. (2) Under appropriate conditions primary hamster and rabbit tracheal epithelial cell cultures synthesize and secrete mucins of large molecular size (>10^6 daltons). The high molecular weight materials are completely resistant to proteoglycan degrading enzymes, they contain N-acetylgalactosamine, N-acetylglucosamine, galactose and fucose as well as sialic acid, but they do not contain mannose. This together with evidence for O-glycosidic linkage of N-acetylgalactosamine to protein provides strong evidence that the secreted materials are indeed mucins.

Further studies provided evidence for the heterogeneity of the mucins produced by the cultures in terms of degree of sulfation, size of the oligosaccharide chains (ranging from 5,000 to 15,000 MW) and binding affinity of the mucins for various lectins. As soon as the studies on the characterization of mucin produced by the tracheal cell cultures are complete this model system will be used to investigate the regulation of mucin biosynthesis and secretion.

Another major research topic pursued by the Cell Biology Group is to elucidate the mechanisms regulating differentiation of the epithelial cells of the conducting airways. It is known from in vivo as well as cell culture studies that this epithelium has the potential for mucociliary as well as epidermoid (keratinocytic) differentiation. Using rabbit cell cultures several factors were established which profoundly influenced tracheal epithelial cell differentiation. Serum promotes keratinocyte differentiation so does Ca^{++} at high concentrations but only when the cultures have reached a state of confluency. On the other hand, mesenchymal factors obtained from 3T3 fibroblasts and retinoids inhibit keratinocyte differentiation and promote mucociliary differentiation. Several important biochemical observations were made. Undifferentiated-

proliferative cells made large amounts of hyaluronic acid. When stimulated to undergo terminal differentiation hyaluronic acid production was drastically reduced. When the rabbit tracheal cells underwent keratinocyte differentiation, their keratin profile changed. The production of 56 kd keratin increased and 54 and 48 kd keratins appeared. Vitamin A inhibited the synthesis of these keratins. The 48 kd keratin appeared to be a particularly good marker for epidermoid differentiation and studies are currently underway to elucidate the molecular mechanisms by which retinoids regulate its expression.

The mechanism of action of retinoid is one of the major research topics of the Cell Biology Group. Retionids have been shown to effectively block the induction of the enzyme ornithine decarboxylase (ODC) by the phorbolester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). This enzyme is the rate limiting step in polyamine biosynthesis and appears to play an important role in biochemical events involved in tumor promotion. The question being asked how and at what level of molecular organization do retinoids inhibit the induction of ODC by TPA. It was shown that retinoids do not interfere with TPA binding nor do they interfere with protein kinase C (the presumed TPA receptor) activity. This raises the possibility that retinoids might interfere with signal transduction from the TPA receptor to the nucleus or directly interfere with transcription. These and other possible mechanisms of the retinoid effects will be examined in the future.

Epithelial Carcinogenesis Group. The Epithelial Carcinogenesis Group has developed a cell culture model of multistage carcinogenesis using tracheal epithelial cells from rats (RTE cells). Several aspects of the work of this group are closely related to research pursued in the Cell Biology Group. The major goals of this work are: (1) To identify and characterize distinct steps in neoplastic transformation of epithelial cells, (2) to study the rates of progression from one preneoplastic stage to the subsequent stage, (3) to identify factors which are important determinates for the rate of progression, and (4) to elucidate cellular and molecular mechanisms involved. This work is being performed in collaboration with several members of the Environmental Carcinogensis Group. One of the problems which was addressed during the past year concerns the propagation and growth kinetics of transformed epithelial cells. The question was asked: what are the characteristics of the cells composing the transformed colony in the RTE cell system? Do all the cells in such colonies, which are presumably of clonal origin, exhibits transformed characteristics? The major feature of transformed RTE cells, which distinguishes them from normal RTE cells, is their capacity to grow under conditions which are nonpermissive for normal tracheal cells and to become "immortal." This transformed cell variant is therefore called enhanced growth (EG) variant. Studies showed that only a small fraction of the cells in the transformed colony has transformed growth characteristics. The majority of the cells in such colonies are terminal, nonreplicating cells. However it was also found that the proportion of "transformed stem cells" increases with time after carcinogen exposure from a frequency which was initially in the order of 10^{-4} to a frequency of 10^{-1}. These and other studies suggest that important qualitative as well as quantitative changes are occurring in the early transformed cell populations.

The early post-initiation phase of transformation also seems to be particularly sensitive to modulators of neoplastic progression. This conclusion was reached because it was found that repeated exposures during this phase of transformation to the tumor promoter TPA enchances the development of the anchorage independent

phenotype (a cellular phenotype which is closely associated with late pre-neoplastic and possibly neoplastic cell variants) during later stages of neoplastic transformation. On the other hand the exposure to the anti-promoting agent retinoic acid (RA) markedly inhibited the formation of transformed colonies even when the exposure to the retinoic acid was limited to only 1 week (the expression-selection time of the transformation studies is 5 weeks). Studies on the sensitivity and response pattern of cells at various stages of transformation may help to define the stages in biological as well as biochemical terms. Interesting differences between normal and stably transformed RTE cells in response to the tumor promoter TPA as well as to retinoic acid are beginning to emerge. Since these agents are known to have profound influences on cell proliferation as well as cell differentiation, these studies may lead us to the relevant dysfunctioning biochemical control mechanisms in various stages of neoplastic transformation. Studies have been initiated which are aimed at identifying important cellular genes which are responsible for the abnormal growth behavior of the transformed RTE cells.

Environmental Carcinogenesis Group. One of the topics of investigation of the Environmental Carcinogenesis Group is the elucidation of the relationship between mutagenesis and carcinogenesis. The majority of carcinogens tested to date have been found to be mutagenes. This has lead to the widely accepted hypothesis that at least one of the key steps in carcinogenesis involves structural damage to DNA and gene mutation. However a number of carcinogens do not appear to cause point mutations (and thus appear not to be "mutagenic" in the narrow sense of the word). The Environmental Carcinogenesis Group has selected a number of such carcinogenic compounds to determine their mechanism of action. Two particularly fascinating carcinogens which have been extensively studied are diethylstilbestrol (DES) and asbestos. Both are important human carcinogens and were tested in the Syrian hamster embryo (SHE) cell system for their ability to cause point mutations, unscheduled DNA synthesis, sister chromatid exchanges, chromosomal abnormalities and morphological transformation. The studies showed that DES (without addition of a metabolic activation system) causes morphological transformation in a dose-dependent fashion but neither causes mutations at two specific genetic loci (HPRT and Na^+/K^+ ATPase), unscheduled DNA synthesis nor sister chromatid exchange. However DES, under the same experimental conditions caused numerical chromosomal abnormalities by affecting spindle microtubule organization and behaved in many respects similar to the well-known aneuploidy-inducer, colcemid. Studies with asbestos fibers, glass fibers and non-fibrous particulates carried out in collaboration with the Pulmonary Pathology Group showed that, similar to *in vivo* tumorigenesis, long and thin fibers, regardless of chemical composition, caused morphological transformation while thick and short fibers are very ineffective in this respect. It was also found that neither the asbestos nor the fiberglass fibers induce measurable point mutations or unscheduled DNA synthesis. However fibers of the appropriate physical dimensions (average length approximately 10 microns and average thickness approximately 1 micron) caused structural and in particular numerical chromosomal abnormalities. Thus these studies suggest that aneuploidy induction may be an important mechanism of transformation and carcinogenesis at least with carcinogens which do not cause measurable structural DNA damage. These studies are been extended to include compounds such as benzene and dioxins and to further explore the possible role of aneuploidy in carcinogenesis.

A newly initiated area of investigation deals with exploration of the molecular basis of the multiphasic development of neoplastic transformation using the SHE

cell system which has been well defined in terms of progression of preneoplastic to neoplastic stages. It was found that normal SHE cells can be neoplastically transformed with polyoma virus DNA but not with Ha-MSV DNA. RSV-DNA was occasionally tumorigenic but the tumor latency was long. In contrast transfection with combinations of viral DNAs (Ha-MSV plus pSVv-myc and RSV plus pSVv-myc) resulted in efficient neoplastic transformation with short tumor latency. When preneoplastic SHE cell lines were transfected with either polyoma Ha-MSV or RSV-DNA rapid conversion to the neoplastic state occurred. These studies thus provide strong support for the role of several oncogenes in the multistep process of carcinogenesis. Future studies will more extensively explore the cooperation of oncogenes and the expression of cellular oncogenes during neoplastic progression in the SHE cell model.

Pulmonary Pathology Group. The overall research objectives of this group are to elucidate pathogenetic mechanisms involved in the development of particle and fiber induced pulmonary fibrosis specifically asbestosis and silicosis. The pulmonary macrophage appears to play a key role in the early phases of tissue damage caused by inhaled fibers. After the deposition of fibers in the air spaces macrophages migrate to the site of deposition and within 48 hrs microscopic lesions develop which are presumed to be the early manifestations of the developing fibrotic reaction. The questions being addressed by the Pulmonary Pathology Group are: what is the nature of the signal responsible for macrophage migration to the site of particle deposition, what factors govern the particle macrophage interaction at the level of the cell membrane and what characterizes, at the tissue level, the epithelial and the mesenchymal response?

A number of in vivo and in vitro studies support the hypothesis that the signal which attracts macrophages to the site of asbestos deposition is a complement derived chemotactic factor (C5A) activated by interaction of asbestos fibers with proteins in the alveolar lining layer. The evidence is as follows: genetically complement deficient mice as well as pharmacologically complement deprived rats have a markedly reduced macrophage response upon inhalation of asbestos. Pulmonary lavage fluid obtained from rats shortly after a brief inhalation exposure to asbestos contains a factor which is chemotactic for pulmonary macrophages. Based on molecular size this factor is similar to activated complement which is a strong chemotactic agent. Chemotactic activity is removed by decomplementation and treatment with EDTA. The chemotactic factor can be generated in vitro by incubation of serum as well as cell free alveolar lavage fluid obtained from normal unexposed rats with asbestos. Studies are planned to further examine this hypothesis, e.g., different particles and fibers will be examined and compared in terms of their capacity (a) to activate complement in vitro and (b) to stimulate marophage migration in vivo. Studies are also planned to examine the effects of complement depletion on the development of a interstitial lung disease.

If macrophages play a key role in the induction of interstitial lung disease by fibers as is currently being assumed, then it is essential that we obtain a better understanding of the factors controlling the interaction between macrophages and fibers. Initial experiments, using erythrocytes as a model to study fiber-membrane interactions, showed that the hemolysis caused by chrysotile involved the binding of the positively charged chrysotile to negatively charge sialic acid residues on the erythrocyte membrane. More recently studies have been carried out with pulmonary macrophages. These have shown, using the lectin wheat germ agglutinin (WGA), that sialic acid (SA) binding sites are

evenly distributed over the macrophage membrane, that chrysotile asbestos as well as carbonyl iron particles bind to the macrophage membrane upon incubation at 40°C (no phagocytosis) and that this binding can be blocked by prior incubation of the macrophages with WGA which occupies the sialic acid groups. It was further shown that wheat germ agglutinin also blocks phagocytosis at 37°C. These data strongly suggest that the sialic acid residues on pulmonary macrophage membranes are essential for binding as well as phagocytosis of positively charged fibers and particles. Current studies are concerned with the pathophysiological and biochemical consequences of particle binding and particle phagocytosis for macrophages themselves as well as for cells exposed to the macrophage products resulting from the particle membrane interaction.

Other studies of the Pulmonary Pathology Group are concerned with deposition and translocation of asbestos fibers in various tissue compartments of the lungs and with the development of early tissue lesions believed to be the "precursors" of the chronic fibrotic reaction characterizing asbestosis. These studies have identified the alveolar duct bifurcations as the most important deposition site with respect to development of fibrotic lung disease. They have further shown rapid translocation of fibers through the epithelium into the interstitium. Within 1 month after a short inhalation exposure to asbestos, increased cellularity and deposition of extracellular matrix materials was observed in the interstitium of the alveolar duct bifurcations. These findings strongly suggest that the alveolar duct region is most likely the area in which pulmonary asbestosis first develops. Other studies indicate that approximately 20% of the fibers initially deposited in the lungs are retained 1 month after a brief exposure and that the longer fibers appear to be selectively retained. It is these remaining fibers which are suspected to play the key role in the interstitial fibrosis, typical of asbestosis, by mechanisms which are unknown at the moment.

Pulmonary Biochemical Group. The Pulmonary Biochemical Group has been interested for many years in the phospholipids of the lung, the so-called pulmonary surfactants, which are essential for pulmonary lung function, preventing collapse of alveoli and distal airways at expiration. It is firmly established that the source of the surfactant are the so-called Type II alveolar cells, epithelial cells which also serve as "reserve cells" for the alveolar epithelium. However the mechanisms regulating surfactant biosynthesis, secretion and removal from the alveolar spaces are presently not known.

Silica dust which is a material of considerable environmental and occupational concern since it can cause chronic obstructive lung disease (silicosis) was found to cause a marked increase in surfactant levels in the lungs of exposed rabbits. This effect of silica provides an opportunity to study the interrelationships between surfactant synthesis, release and removal and as a means to disturb the normal equilibrium and clarify some of the pathogenetic mechanisms involved in silica induced pulmonary disease. The initial research efforts devoted to this project were designed (1) to develop methodologies to quantitative intra- and extracellular surfactant pools in the lungs of rabbits, and (2) to characterize and quantitate the changes in intra- and extracellular surfactant resulting from a large exposure to silica. Time and dose-response relationships were established 1 month after a single dose of silica, administered by intratracheal instillation. The intracellular pool was increased 80-fold and the extracellular surfactant pool was increased 29-fold above normal. Using ^{3}H-palmitate as a phospholipid precursor, measurements of the rates of surfactant synthesis,

transfer to the extracellular pool and turnover in the extracellular pool, were conducted. These studies showed that biosynthesis was increased about 10-fold, transfer to the extracellular pool about 4-fold and extracellular turnover approximately 2-fold. This suggests that the primary (though not the only) disruption of the surfactant system by silica may be resulting from a massive stimulation of surfactant synthesis which neither the secretory mechanisms nor the extracellular removal mechanism are able to cope with. Further studies will be conducted with isolated Type II cells from normal and silica exposed animals to analyze the observed phenomena further.

In collaboration with the members of the Cell Biology Group, another project has been pursued, which is aimed at the elucidation of the function of the so-called "Clara cell", an epithelial cell prominent in the distal airways. This cell is believed to be a secretory cell based on ultrastructural evidence. It is a cell rich in cytochrome P-450 and is highly active in the metabolism of a variety of xenobiotics. The Clara cell is also believed to function as a "reserve" cell of the epithelium of the terminal bronchioles. Because of the fact that Clara cells are a minority cell population (about 8% of the cells in the lung digest are identifiable as Clara cells) methods were developed to isolate Clara cells from enzymatic lung digests. Using continuous density percoll gradients purification of Clara cells to 40% was achieved. This cell mixture was subsequently submitted to centrifugal elutriation which resulted in cell preparations containing 75-85% Clara cells. These cells are viable as judged by dye exclusion, they survive in culture for at least several days and are active biosynthetically. Upon incubation with ^{35}S-methionine or a cocktail of ^{14}C-labelled amino acids, the cells can be shown to synthesize and release into the medium a great number of proteins. The major radiolabelled protein released into the medium has a molecular weight of about 10,000 daltons as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. One major task for the immediate future is to determine whether or not this protein synthesized by the Clara cells *in vitro* is an important secretory product produced *in vivo*. Experiments are in progress to compare the proteins released by Clara cells with those produced by other epithelial cells isolated from the lungs of rabbits. These experiments will allow us to identify which of the products released by Clara cells are Clara cell specific.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES-25001-07 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Mutagenesis in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	M. Oshimura	Expert	LPFT, NIEHS
	N. Tanaka	Visiting Fellow	LPFT, NIEHS
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Environmental Carcinogenesis Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Most chemical carcinogens induce DNA damage and are mutagenic at specific genetic loci; however, certain carcinogens (including the human carcinogens diethylstilbestrol, asbestos, arsenicals and benzene) usually do not induce gene mutations. We have examined the activity of these chemicals, proposed as carcinogenic but not mutagenic, to induce morphological transformation, gene mutations and chromosome mutations in Syrian hamster embryo cells in culture. Diethylstilbestrol (DES) induced cell transformation in the absence of mutations at specific genetic loci but was an inducer of aneuploidy by nondisjunction. DES was found to disrupt spindle microtubule organization in the cells and to cause chromosome loss and gain. Cell transformation and aneuploidy induction by DES were similar in terms of dose response and cell-cycle dependence. Furthermore, colcemid, a well known inducer of nondisjunction was also found to induce neoplastic transformation of cells in culture, confirming that aneuploidy induction is a potential mechanism for cell transformation. The mechanism of action of two other human carcinogens, asbestos and arsenic, were studied. Asbestos and other mineral fibers, including fiberglass, induced cell transformation in a dose dependent manner. The ability of fibers to induce cell transformation depended on fiber dimension, i.e., long, thin fibers were most potent. These findings indicate that this experimental system is an useful model to study the mechanism of fiber-induced tumorigenesis since similar results are found for fiber size dependence for mesothelioma induction in animals. An excellent correlation was observed between the induction of cell transformation and chromosome changes, including numerical and structural aberrations, suggesting that asbestos causes cell transformation by affecting chromosome stabilty. Arsenic also induced cell transformation and caused chromosome aberrations. The results with different carcinogens indicate that a significant effect of carcinogens is on chromosome number and structure and that these changes are important in the genesis of carcinogenesis.

PROJECT DESCRIPTION

A significant number of known chemical carcinogens have been shown to be activated to reactive, electrophilic derivatives which directly interact with DNA and cause mutations at specific genetic loci. This has led to the general hypothesis that carcinogenesis results from gene mutations generated by carcinogens damaging DNA. This hypothesis is supported by several lines of evidence and is generally accepted as the mechanism of action of many chemical carcinogens. However, some chemical carcinogens are inactive in short-term tests for DNA damage or specific locus mutations which may indicate alternative mechanisms for chemically-induced cancers. While these chemicals may be the exceptions to the correlation between carcinogenesis and mutagenesis and limited in number, many of these substances are known human carcinogens (e.g., asbestos, benzene, arsenicals, and diethylstilbestrol) and therefore, a better understanding of their mechanism of action is highly significant to carcinogenesis in humans.

Our approach to understanding the role of mutagenesis in carcinogenesis has been to develop an experimental model which can detect different stages in the neoplastic development of cells in culture and to compare the abilities of chemicals to induce neoplastic transformation and different types of mutational changes concomitantly in the same cells. The objectives of this research are:

(1) To determine the activity in the Syrian hamster embryo cell transformation assay of known human and/or animal carcinogens with emphasis on chemicals which are inactive in certain mutational assays such as the Ames test.

(2) To correlate the ability of chemicals to induce cell transformation, gene mutations, and chromosome mutations.

(3) To understand the mechanism of action of chemicals which do not induce gene mutations but induce cell transformation.

(4) To determine the mechanism by which tumor promoters enhance or induce cell transformation.

The experimental system for these studies is the Syrian hamster embryo (SHE) cell transformation assay. Early passage, normal, diploid SHE cells, when exposed to chemical carcinogens, undergo a progressive series of changes resulting in the neoplastic transformation of the cells. Various phenotypic markers can be used to quantitate early, preneoplastic changes (e.g., morphological transformation) and later stages associated with the neoplastic conversion of the cells (e.g., anchorage-independent growth). In this project, the mechanism of the initial stage in this neoplastic progression is studied by comparing the ability of different chemicals to induce morphological transformation and mutations in the same cells. Three types of mutational activity are measured: (1) gene mutations at two specific genetic loci (HPRT and Na^+/K^+ ATPase); (2) chromosome aberrations (breaks, fragments, and rearrangements); and (3) aneuploidy, i.e., numerical chromosome aberrations. DNA damage in the SHE cells is also measured by an unscheduled DNA synthesis (UDS) assay.

Our initial studies with diethylstilbestrol (DES) indicated that cell transformation could be induced in the absence of detectable mutations at two specific genetic loci. No DNA damage by DES was detected by UDS assay or by sister

chromatid exchange assays. However, DES was found to be an effective inducer of aneuploidy probably via nondisjunction. We have now shown that DES affects microtubule organization in SHE cells and that spindle microtubules are very sensitive to DES. This provides a biochemical basis for DES-induced nondisjunction and possibly cell transformation. Colcemid, a well known inducer of nondisjunction was tested for its ability to induce morphological and neoplastic transformation of the cells and was found to be active in these assays and to resemble DES in terms of its dose-response curves for cell transformation and aneuploidy induction.

A second pathway for DES-induced carcinogenicity was suggested by studies on the effects on cells of DES in the presence of an exogenous metabolic activation system using rat liver microsomes. Under these conditions DES is metabolized to potentially reactive intermediates and induces UDS, gene mutation and enhanced cell transformation. Studies are in progress to determine which metabolites of DES are active in directly damaging DNA.

The abilities of chrysotile and crocidolite asbestos, glass fibers of differing dimensions, and non-fibrous mineral particulates to induce morphological transformation of Syrian hamster embryo cells in culture were compared. Chrysotile and crocidolite asbestos induced morphologically transformed colonies which were indistinguishable from transformed colonies observed following treatment with known chemical carcinogens. A linear, dose-dependent increase in the frequency of transformed colonies was observed. The slope of the dose-response curve on a log-log scale was ~1, which is consistent with a one-hit mechanism for their induction. The transforming potency of chrysotile asbestos was decreased by milling of the fibers but not by extraction with an organic solvent. Chrysotile asbestos was nearly twice as potent in inducing morphological transformation as crocidolite asbestos. Glass fibers were also very active in this assay. Thin glass fibers with an average diameter of 0.1 - 0.2 μm were as active as chrysotile asbestos. In contrast, two non-fibrous particulates, alpha quartz and Min-U-Sil, were inactive over the same concentration range employed for the fibrous dusts; however, both were active at higher doses.

The effect of varying fiber dimensions on induction of morphological transformation was examined with glass fibers. When compared on a per weight basis, thick glass fibers (average diameter 0.8±0.6 μm) were 20-fold less potent than thin fibers (average diameter 0.13±0.005 μm) in inducing cell transformation. When the average fiber length of thin glass fibers was reduced from 9.5 μm to 1.7 μm by milling the fibers in a mortar and pestle, a 10-fold decrease in transforming activity resulted. When the average fiber length was reduced to 0.95 μm, transforming ability was totally absent. The cytotoxic potencies of the various mineral dusts correlated with their transforming potencies. The varying ability of the mineral dusts to induce cell transformation *in vitro* is similar to their abilities to induce mesotheliomas *in vivo*. Thus, this system provides an unique model for studying the mechanism of mineral fiber tumorigenesis and for comparing the relative risks of various mineral dusts.

Cytogenetic effects of chrysotile asbestos on Syrian hamster embryo cells *in vitro* were investigated at doses which induced morphological and neoplastic

transformation, but failed to induce measurable gene mutations in the cells at two genetic loci. Chrysotile asbestos treatment of the cells caused only a marginal increase in sister chromatid exchange levels at only the highest dose tested (2.0 $\mu g/cm^2$ of culture surface), but significantly induced other chromosome changes in a dose-dependent manner. Up to 50% of the cells had chromosome abnormalities in number or structure following treatment with 2.0 $\mu g/cm^2$ asbestos for 48 hr. Numerical chromosome changes were the most pronounced abnormalities although significant increases in metaphases with other chromosome aberrations (breaks, fragments, exchanges and/or dicentrics) and cells with binuclei or micronuclei were also observed. A linear relationship was observed between the incidences of cells with tetraploid metaphases and binucleated cells, suggesting that binucleation and tetraploidy are related. Cytogenetic effects of other mineral dusts were also tested 48 hr following treatment at a concentration of 2.0 $\mu g/cm^2$. Crocidolite asbestos was less potent than chrysotile asbestos in its ability to induce cell transformation and cytogenetic damage. Treatment of the cells with thin glass fibers (code 100) was also able to induce cell transformation and cytogenetic effects, but thick glass fibers (code 110) were much less potent for both end-points. Milling of the thin glass fibers decreased the length of the fibers and abolished their ability to induce cell transformation and cytogenetic effects. Non-fibrous alpha-quartz induced neither cell transformation nor cytogenetic effects at the dose of 2.0 $\mu g/cm^2$. The results indicate that the physical characteristics of the fibers determine their ability to induce cell transformation and their ability to induce chromosome mutations suggesting a possible mechanistic relationship.

Sodium arsenite and sodium arsenate were observed to induce cell transformation with the trivalent form being 10-20 fold more potent than pentavalent arsenic. While these arsencials were inactive in inducing gene mutations, both were very active as clastogens and inducers of sister chromatid exchanges. No numerical chromosomes abnormalities were induced by these carcinogens with the exception of endo-reduplication. Thus, these carcinogens appear to act by causing structural rather than numerical chromosome changes.

Two tumor promoters, phenobarbital and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were tested as inducers of morphological transformation and found to be inactive. TPA also failed to induce any chromosome mutations (structural or numerical). Thus, induction of cell transformation and aneuploidy is unrelated to the tumor-promoting activity of the chemicals examined.

Future studies will examine the mechanism of action of benzene, sodium bisulfite, diethylhexylphthalate and 2,3,7,8-tetrachlorodibenzodioxin. Preliminary evidence exists that at least the first three compounds are active in the cell transformation assay. Since these are suggested as inactive gene mutagens, we hope to extend our observations on the role of chromosome mutagens in cell transformation and carcinogenicity. In addition, transformed cells will be examined for specific chromosome changes and these will be correlated with molecular studies of the transformed phenotypes of these cells. The mechanism by which asbestos and mineral fibers and other chemicals induce aneuploidy will be examined. Attempts to develop genetic assays for measuring the ability of a chemical to induce aneuploidy and gene amplification will be made.

PUBLICATIONS

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Elmore, E., Kakunaga, T., and Barrett, J. C.: Comparison of the spontaneous mutation rates of normal and chemically transformed human skin fibroblasts. Can. Res. 43: 1650-1655, 1983.

Degen, G. H., Wong, A., Eling, T. E., Barrett, J. C., and McLachlan, J. A.: Peroxidative metabolism of diethylstilbestrol in Syrian hamster embryo fibroblast cell cultures. Can. Res. 43: 992-996, 1983.

Barrett, J. C., Hesterberg, T. W., and Thomassen, D. G.: Use of cell transformation systems for carcinogenicity testing and mechanistic studies of carcinogenesis. Pharmacol. Rev., In Press, 1984.

Barrett, J. C., and Thomassen, D. G.: Use of quantiative cell transformation assays in risk estimation. In Vouk, V. B., Butler, G. C., Hoel, D. G., and Peakall, D. B. (Eds.), Methods of Estimating Risk in Human and Chemical Damage in Non-Human Biota and Ecosystems, SCOPE, SGO, SEC 2, IPCS Joint Symp. 3, John Wiley and Sons, New York, In Press, 1984.

Tsutsui, T., Maizumi, J. A., McLachlan, J. A., and Barrett, J. C.: Possible role of aneuploidy induction and cell transformation diethylstilbestrol: A possible chromosomal mechanism in carcinogenesis. Can. Res. 43: 3814-3821, 1983.

Barrett, J. C., Thomassen, D. G., and Hesterberg, T. W.: Role of gene and chromosomal mutations in cell transformation. Ann. N. Y. Acad. Sci. 407: 291-300, 1983.

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Tsutsui, T., Degen, G. H., Schiffman, D., Wong, A., Maizumi, H., McLachlan, J. A., and Barrett, J. C.: Dependence on exogenous metabolic activation for induction of unscheduled DNA synthesis in Syrian hamster embryo cells by diethylstilbestrol and related compounds. Can. Res. 44: 184-189, 1984.

Tsutsui, T., Maizumi, H., and Barrett, J. C.: Colcemid-induced neoplastic transformation and aneuploidy in mammalian cells in culture. Carcinogenesis 5: 89-93, 1984.

Hesterberg, T. W., and Barrett, J. C.: Dependence of asbestos and mineral dust-induced transformation of mammalian cells in culture on fiber dimension. Can. Res. 44: 2170-2180, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25002-07 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

In Vitro Carcinogenesis and Promotion Studies with Respiratory Tract Epithelium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: P. Nettesheim Chief LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Epithelial Carcinogenesis Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project No. Z01 ES 25023-01 entitled "Studies on the Mechanism of Neoplastic Development in Airway Epithelial Cells."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 25007-06 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Deposition of Inhaled Particles and Pathogenesis of Initial Pulmonary Lesions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: A. R. Brody Research Biologist LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Pulmonary Pathology Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
Study continued under Project No. Z01 ES 25024-01 entitled "Pathogenesis of Early Pulmonary Lesions Induced by Inhaled Inorganic Particles."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 25008-06 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Macrophage Accumulation and the Initial Chemotactic Stimulus Activated by Asbestos

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. R. Brody Research Biologist LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Pulmonary Pathology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project No. Z01 ES 25025-01 entitled "Asbestos Activation of Complement-Dependent Chemotactic Factors for Macrophages."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25009-05 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Interactions with Chrysotile and Crocidolite Asbestos

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. R. Brody Research Biologist LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

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INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project No. Z01 ES 25026-01 entitled "Interactions of Inorganic Particles with Pulmonary Cell Membranes."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25014-02 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Cell-Cell and Cell-Substratum Interactions in Cell Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. M. Jetten Senior Staff Fellow LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Cell Biology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project No. Z01 ES 25021-01 entitled "Differentiation and Differentiative Functions of Tracheal Epithelial Cells."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 25017-02 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of Proliferation and Its Relation to Cellular Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. M. Jetten Senior Staff Fellow LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Cell Biology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Study continued under Project No. Z01 ES 25022-01 entitled "Study of the Molecular Mechanisms of Action of Retinoids."

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25020-02 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of the Pulmonary Surfactant System and its Modification by Toxic Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. E. R. Hook	Research Chemist	LPFT, NIEHS
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	L. B. Gilmore	Biologist	LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Biochemical Pathology Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Pulmonary surfactant is a complex mixture of lipids and proteins that prevents collapse of the alveoli and distal airways at low lung volumes. In the lungs surfactant exists within two major compartments referred to, respectively, as the intracellular and extracellular pools. The most potent inducer of the pulmonary surfactant system reported appears to be silica dust. The objectives of this work are to elucidate mechanisms through which silica stimulates surfactant production in the lungs. Extracellular surfactant from the lungs of rats was obtained by bronchoalveolar lavage. Intracellular surfactant was isolated from lavaged lungs by density gradient centrifugation of the homogenized tissue. Intra- and extracellular pools of surfactant phospholipids were 0.69±0.27 mg/pair of lungs and 1.21±0.09 mg/pair of lungs, respectively. Intratracheal injection of silica produced marked changes in both the intracellular and extracellular pools of surfactant, an effect that was both time- and dose-related. Twenty-eight days following a single 50 mg dose of silica, the intra- and extracellular pools of surfactant were increased 80-fold and 29-fold, respectively. Expansion of the surfactant pools could be achieved through alterations in the dynamic equilibrium that under normal circumstances exists between the intracellular and extracellular compartments. This hypothesis was examined *in vivo* using ^{3}H-palmitate as a phospholipid precursor. Biosynthesis of surfactant phospholipids was increased approximately 10-fold but transfer to the extracellular pool increased only 4-fold thus accounting for the expansion of the intracellular pool of surfactant. Although secretion increased 4-fold the turnover of phospholipids in the extracellular pool increased only 2-fold thereby leading to expansion of the extracellular pool. These studies demonstrate that silica causes a large increase in the biosynthesis of surfactant phospholipids and that the expansion of the intracellular and extracellular pools is due to imbalances in the dynamic equilibrium between the two compartments.

PROJECT DESCRIPTION

Our studies indicate that silica is the most highly effective stimulator of the pulmonary surfactant system yet discovered. The mechanisms through which this stimulation is achieved are not known although, potentially, such mechanisms could prove extremely important in understanding the processes through which the pulmonary surfactant system is regulated and controlled. The objectives of this research are to elucidate the mechanisms through which silica increases phospholipids of the surfactant system.

Initial studies have focused on characterization of the response of the surfactant pools to silica and examination of other phospholipid compartments in the lungs. All phospholipid compartments within the lungs of rats increase in response to intratracheal instillation of silica, although the largest increases are seen in the intracellular and extracellular pools of pulmonary surfactant. Increases in the intracellular and extracellular pools of surfactant have been as much as 80-fold and 29-fold, respectively. No other agent has been reported to stimulate the intracellular pool of surfactant previously and no other agent has been reported to stimulate the extracellular pool to such a large extent. Maximum increases reported previously have been an order of magnitude lower than those obtained with silica.

The literature indicates that under normal circumstances the intracellular and extracellular pools of pulmonary surfactant are in a state of dynamic equilibrium in which biosynthesis of phospholipids and their incorporation into the intracellular pool is balanced by the transfer of phospholipids from that pool to the extracellular pool and that this transfer, in turn, is balanced by removal of phospholipids from the extracellular pool thus leading to static pool sizes. Expansion of the intracellular and extracellular pools of surfactant could arise through imbalances in the various rate processes that normally contribute to and maintain a state of dynamic equilibrium. This hypothesis was tested by injecting ^{3}H-palmitate into rats and measuring the rates at which phospholipids were synthesized and transferred into the intracellular pool of surfactant, the rates at which phospholipids were transferred from the intracellular to extracellular pools, and the turnover rates of phospholipids in the extracellular pools. All of these factors were elevated following intratracheal injection of silica (10 mg) into the lungs of rats. However, biosynthesis of surfactant phospholipids increased approximately 10-fold but transfer to the extracellular pool increased only 4-fold thus accounting for the expansion of the intracellular pool. Although secretion increased 4-fold, the turnover of phospholipids in the extracellular pool increased only 2-fold thereby leading to expansion of the extracellular pool. Our hypothesis concerning imbalances in rate processes leading to expansion of pool sizes appears to be true.

In the subsequent year we have two major objectives. First, we will study Type II cells isolated from the lungs of silica-treated rats and determine if their phospholipid synthesizing abilities are different from those of Type II cells from the lungs of normal rats. Preliminary investigations indicate the appearance in the lungs of a new variety of Type II cell in response to silica. Second, we will investigate the direct interactions between isolated Type II

cells and silica and the effect of those interactions on surfactant biosynthesis and secretion. Direct interactions between silica and the plasma membrane of Type II cells could lead to stimulation of phospholipid biosynthesis possibly through the release of prostaglandins or other messengers or through the direct action of silica on receptors present in the membrane.

PUBLICATIONS

Hook, G.E.R., Gilmore, L.B., and Talley, F.A.: Multilamellated structures from the lungs of patients with pulmonary alveolar proteinosis. Lab. Invest. In press.

Hook, G.E.R., Ortner, M.J., Chignell, C.F., Spalding, J.W., and Tombropoulos, E.G.: Investigation of phospholipids of the pulmonary extracellular lining by using electron paramagnetic resonance: the effects of phosphatidylglycerol and unsaturated phosphatidylcholines on the fluidity of dipalmitoylphosphatidylcholine. Biochem. J. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 25021-01 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Differentiation and Differentiative Functions of Tracheal Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. M. Jetten	Sen. Staff Fellow	LPFT, NIEHS
Others:	J. Rearick	Staff Fellow	LPFT, NIEHS
	H. Smits	Visiting Fellow	LPFT, NIEHS
	M. E. Porter	Biol. Lab. Tech.	LPFT, NIEHS
	M. Deas	Chemist	LPFT, NIEHS

COOPERATING UNITS *(if any)*

Continuation of Project No. Z01 ES 25014-02

LAB/BRANCH

Laboratory of Pulmonary Function and Toxicology

SECTION

Cell Biology Group

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

For the study of the regulation of mucin synthesis and secretion hamster tracheal epithelial cell cultures grown on collagen gels appear to provide us with a good model system. Cells were radiolabelled with ^{3}H-glucosamine and/or ^{35}S-sulfate and their secretory products were characterized. Mucins were identified on the basis of their high molecular weight and complete resistance to proteoglycan-degrading enzymes, ruling out proteoglycans. Labelling with several monosaccharide precursors and analysis of the high molecular weight products by strong acid hydrolysis or neuraminidase treatment showed the presence of sialic acid but not mannose. β-Elimination released oligosaccharides with the conversion of most of the N-acetylgalactosamine into N-acetylgalactosaminitol, indicating that the oligosaccharides are O-glycosidically linked with protein. This mucin has been further characterized via β-endogalactosidase treatment and it was shown that R-GlcNacβ1-3Galβ1-R linkages are present. This finding is supported by lectin affinity chromatography on DSA-agarose gels. Studies on the action of retinoids and monensin on mucin secretion are in progress. For the study of the regulation of differentiation rabbit tracheal epithelial cell cultures are used as a model system. These cells undergo terminal differentiation into squamous, cornifying cells when reaching confluency. This process is inhibited by retinoids and promoted by calcium and serum. Several biochemical changes accompany this change in phenotype. Undifferentiated cells produce large amounts of hyaluronic acid whereas in keratinizing cells the synthesis of hyaluronic acid is very much reduced. Cells undergo quantitative as well as qualitative changes in keratin proteins as shown by 2-D electrophoresis and by immunoblot analysis using monoclonal antibodies. A 48 kd keratin appears to be a good marker for this differentiation. Differentiation into mucin secretory cells can be induced when cells are grown on collagen gels in the presence of retinoic acid. Retinoic acid appears to have a key role in the control of differentiation.

PROJECT DESCRIPTION

Our group is engaged in the study of the biological functions of airway epithelium. One effort focuses on the regulation of mucin synthesis and secretion, the other on the regulation of differentiation of the epithelial cell population. These functions have been previously studied in whole animals and in organ culture systems. These studies have provided important information about certain characteristics of these cells. Although these systems have certain advantages, the amount of extraneous tissue makes it difficult to study the regulatory mechanisms underlying the control of epithelial differentiation and mucin production at the molecular level. The recent development of epithelial cell culture systems permits us to study factors regulating the commitment of epithelial cells to specific pathways of differentiation. It also makes possible studies of the regulation of several differentiative functions such as the production of mucin on a molecular level.

Regulation of Mucin Synthesis and Secretion

Mucin glycoproteins have been isolated from the conducting airways of several species and their composition partially characterized. However, little is known about the factors that regulate its synthesis and secretion. Recently, our laboratory has established an in vitro culture system of hamster tracheal epithelial cells. Morphological observations have shown that these cultures contain under appropriate conditions cells with secretory granules which based on histological procedures contain neutral glycoproteins. These cells form a promising model system to study the regulation of mucin production. The advantage of such an in vitro system are clear: one can study the action of exogenous regulators on mucin production directly. The specific objectives are:

1. to demonstrate mucin production in vitro according to biochemical criteria.
2. to structurally characterize mucin oligosaccharides to serve as a reference point for comparison in studies using modulators of mucin production.
3. to develop a rapid assay for mucin, employing the information gained above.
4. to use this system to study the regulation of mucin production (synthesis, intracellular processing and secretion, differentiation) using modulators such as monensin and retinoids.

Methods for isolating epithelial cells from the tracheal lumen, and for studying the growth and morphological characteristics of these cells, have been established previously in this laboratory. During culture, the cells are metabolically labelled by adding radioactive precursors of mucin, monosaccharides, sulfate and amino acids, to the growth medium. The secreted, radiolabelled products are harvested with the medium, while intracellular intermediates can be obtained from the cells by detergent solubilization. Characterization of these fractions is carried out using gel filtration and ion exchange chromatography and specific enzymatic and chemical degradation procedures which are diagnostic either for general classes of glycoconjugates or for specific monosaccharides and/or glycosidic linkages. Column chromatography using resins containing immobilized sugar-binding proteins (lectin affinity chromatography) which also

discriminates on the basis of mono- or oligosaccharide content and linkage, are used to develop an assay for mucins.

We have shown that hamster tracheal epithelial (HTE) cells secrete radiolabelled mucin into the medium based on the following criteria. As judged by Sepharose CL-4B gel filtration chromatography about one-third of the ^{3}H-glucosamine labelled macromolecules exhibit a molecular weight of larger than 10^{6} daltons.

This high molecular weight material is completely resistant to the action of several proteoglycan-degrading treatments including the enzymes hyaluronidase, chondroitinase ABC, heparinase and degradation by nitrous acid. Proteoglycans are the glycoconjugates most likely to contaminate the high molecular weight material. DEAE-Sephacel anion exchange chromatography also is inconsistent with the presence of proteoglycans.

By labelling with appropriate monosaccharide precursors, N-acetylgalactosamine, N-acetylglucosamine, galactose and fucose were shown to be present in the high molecular weight secreted material by the technique of strong acid hydrolysis while sialic acid was demonstrated by means of susceptibility to neuraminidase. Mannose was shown not to be present. The five monosaccharides found are common components of mucin-type glycoproteins.

Upon reductive β-elimination, all of the incorporated radioactivity is released as lower molecular weight oligosaccharides with the conversion of most of the N-acetylgalactosamine to N-acetylgalactosaminitol, indicating that this monosaccharide was originally involved in O-glycosidic linkage with protein. In summary, HTE cells secrete material which is mucin, based on its high molecular weight, resistance to proteoglycan-degrading treatments, sugar composition and type of glycosidic linkage.

The heterogeneity of the mucin was established by determining the extent of sulfation, size and composition of the oligosaccharide chains. Radioactive sulfate can be incorporated into mucin oligosaccharides but only into about one-third of the mucin molecules as shown by separation of sulfate-rich and relatively unsulfated fractions on DEAE-Sephacel chromatography. The free oligosaccharides themselves show a broad size range from 5,000 to 15,000 MW as assessed by gel filtration chromatography. The oligosaccharides are at least partially digested by an endo-β-galactosidase from E. freundii which cleaves oligosaccharide chains at the sequence R-GlcNAcβ1-3Galβ1-R to yield smaller oligosaccharides. This enzyme will have great utility in further sequence analysis of mucin oligosaccharides. Finally the mucin shows heterogeneity of binding to several lectin affinity columns specific for either N-acetylgalactosamine, N-acetylglucosamine or galactose. Another lectin column, wheat germ agglutinin-agarose shows promise as the basis of a specific and rapid assay for HTE mucin, since 100% of the mucin binds and can be eluted with 1.0 M N-acetylglucosamine, the haptene sugar.

We have shown that hamster tracheal epithelial cells in in vitro culture synthesize and secrete mucin. These glycoproteins are well on their way to being characterized and a rapid assay for these molecules is under development.

This gives us an excellent model system to study the mechanism(s) by which the synthesis and secretion of mucins are regulated. We have begun to investigate the effect of the ionophore monensin on the synthesis, secretion and glycosylation of mucins. In other systems, this drug which partially equilibrates Na^+/K^+ gradients has been shown to cause extensive vacuolization of the Golgi apparatus, the principle site of glycosylation within the cell. This leads to complete inhibition of glycoprotein secretion at concentrations which do not affect protein synthesis and intracellular accumulation of partially glycosylated material. Thus by characterizing structurally any such accumulated material inside HTE cells, it should be possible to define a "monensin-sensitive" step as a potential regulatory mechanism for mucin synthesis presumably by means of changing intracellular Na^+/K^+ levels. This form of regulation could be a component of the action of any inhibitor or stimulator of secretion, including classical secretagogues. The HTE cell culture system can be used to study direct effects upon glycosylation and other biosynthetic and secretory steps by neurohormones, mediators of hypersensitivity and drugs. The effect of these agents on the structure and function of airway secretions has only begun to be examined.

The other line of investigation is to examine the role retinoids play in the regulation of differentiation of airway epithelial cells. Our hypothesis is that retinoids promote normal differentiation of hamster tracheal epithelial cells, thereby indirectly increasing mucin production. Experiments are underway to grow the tracheal cells under vitamin A-deficient conditions in order to test this hypothesis.

Regulation of Differentiation

It has been well established that the epithelial cells of the conducting airways can follow two different pathways of differentiation: (a) into mucin-secreting and ciliated cells, and (b) into squamous keratinizing cells. Retinoids appear to be one of the factors that regulate this differentiation. However, little is known about its mechanism and other factors that might be involved. A rabbit tracheal epithelial culture system has been developed in this laboratory. These cells appear undifferentiated and are able to grow in serum-free, defined medium. This system is an excellent model system to determine what factors are important in controlling differentiation and by what mechanism. Our specific objectives are:

1. to characterize the system by morphological criteria.
2. to develop markers for normal and abnormal differentiation (mucins, keratins, cell surface components).
3. to determine the factors that are important in the regulation of differentiation (substratum, mesenchymal factors, retinoids).
4. to determine the molecular mechanism(s) by which normal and abnormal differentiation are regulated.

For the identification of mucin production and its characterization similar methods are employed as for the mucin produced by hamster cells. For the characterization of intermediate filaments cells were labelled with ^{35}S-methionine and keratins were extracted in high salt concentrations and

detergent. The enriched preparation of keratins was examined by one- and two-dimensional electrophorsis. The identity of the keratins was established by limited proteolytic digestion (Cleveland degradation) and by immunoblot analysis using monoclonal antibodies against keratins.

Growth of rabbit tracheal epithelial cells can be divided into several stages. In the exponential phase cells rapidly proliferate (19 hrs doubling time), have a rounded-up morphology and can easily be subcultured. When reaching confluency cells undergo terminal differentiation into squamous, keratinizing cells and cornified envelopes are formed.

Serum promotes the squamous cell phenotype and enhances cornified envelope formation. High calcium concentrations have little effect on cell proliferation during the exponential phase but stimulate keratinization when cells reach confluency. Conditioned medium from 3T3 fibroblasts inhibits squamous cell formation and stimulates proliferation, indicating that mesenchymal factors influence differentiation and proliferation of epithelial cells. Retinoids inhibit the formation of the squamous cell phenotype and of cornified envelopes. This inhibition occurs at very low concentrations of retinoic acid (10^{-10} M).

Several biochemical changes accompany terminal squamous differentiation. Undifferentiated cells produce large amounts of hyaluronic acid but few other proteoglycans. When cells undergo terminal differentiation the secretion of hyaluronic acid is dramatically reduced. Changes in the synthesis and release of extracellular matrix components may play an important regulatory role in the formation of the squamous cell phenotype.

We have established that rabbit epithelial cells express keratin intermediate filaments but not vimentin. Keratins were identified by one- and two-dimensional electrophoresis. The identity of the individual keratins could be confirmed by peptide maps using Cleveland degradation and two-dimensional electrophoresis. Differentiation is accompanied by changes in the expression of keratins. The major changes are an increase in the expression of a 56 kd keratin and the appearance of 54 and 48 kd keratins. These changes in keratins are prevented by retinoid treatment. The changes in keratin patterns are confirmed via immunoblot analyses using two monoclonal antibodies AE1 and AE3. The 48 kd keratin appears to be an excellent marker for differentiation. The immunoblot is a rapid assay to monitor differentiation.

When cells are cultured on collagen gels in the presence of conditioned medium and retinoic acid, cells are formed that contain secretory granules by transmission electron microscopy. In the absence of retinoic acid none of the cells contain secretory granules and have instead a lot of tonofilaments and desmosomes. Production of mucin was established using the same criteria as for hamster epithelial cells. Mucin production by cells grown in the presence of retinoic acid and conditioned medium has been demonstrated and the mucin has been partially characterized.

Our results indicate that tracheal epithelial cells cultured in vitro can undergo two pathways of differentiation. Depending on the conditions cells can

undergo differentiation into squamous, keratinizing cells or into mucin-secreting cells. In order to study the mechanism by which differentiation is regulated, biochemical characterization of the cells and establishment of differentiative markers is a necessity. We have shown that the synthesis of proteoglycans is altered during squamous cell differentiation and that the substratum is important in the control of differentiation into mucin-secreting cells. To examine the role of the cell surface in the regulation of differentiation, we are determining the changes in cell surface glycoproteins. Some of these changes may function as differentiative markers, whereas others may play a role in the control of differentiation.

Retinoids appear to play a key role in the control of differentiation. There are several possibilities how these compounds exert their action. One hypothesis proposes that retinoids alter directly or indirectly gene expression. The keratin protein of 48 kd appears to be a good marker for differentiation and its expression is under the control of retinoic acid. Whether this control occurs at the transcriptional level has to be determined. Whether retinoic acid acts on the mRNA specific for this keratin is being determined.

PUBLICATIONS

N/A

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25022-01 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Molecular Mechanisms of Action of Retinoids

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. M. Jetten	Senior Staff Fellow	LPFT, NIEHS
Others:	J. E. Shirley	Biological Lab. Tech.	LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Cell Biology Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Retinoids affect many biological and biochemical properties of eukaryotic cells. Their mechanism of action is unclear. One possibility is that they alter gene expression either indirectly or directly via the mediation of the binding protein and interaction with the chromatin. The induction of ornithine decarboxylase (ODC) activity by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) appears to be a good tool to study the mechanism of action of retinoids. Retinoids can affect the induction of this enzyme at several levels. We have no evidence that ODC activity is affected by retinoids at a post-translational level. Transglutaminase appears not to be involved and no evidence of an inhibitory substance activated or induced by retinoic acid was detected. Retinoids could prevent ODC induction by blocking the interaction of TPA with its receptor. We have ruled out this possibility since retinoic acid does not compete with the binding of ^{3}H-PDBu. We have demonstrated that this receptor, which appears to be the protein kinase C, is causal in inducing ODC, since diacylglycerols are able to induce its activity. Retinoic acid does not interfere with the protein kinase C activity. These resultsindicate that retinoic acid acts at a step after the activation of protein kinase C. This could be by interfering with the process of transmitting the signal from protein kinase C to the nucleus or by blocking transcription directly via binding to specific sites on the chromatin. The structure-function relationships of the ability of retinoids to inhibit ODC induction and the capacity to bind to the binding protein suggest a role for these proteins.

(Continuation of Project No. Z01 ES 25017-02 LPFT)

PROJECT DESCRIPTION

Retinoids, analogs of vitamin A, induce many biological and biochemical changes in eukaryotic cells. These compounds have been shown to inhibit tumor promotion in carcinogenesis and alter cellular proliferation and differentiation. The mechanism(s) by which retinoids effect these processes is unknown. In order to study the mechanism by which retinoids act we have chosen the induction of ornithine decarboxylase (ODC) by the phorbol ester tetradecanoyl-phorbol-13-acetate (TPA) as a tool for the following reasons: (a) The induction of ODC by TPA occurs via an increased expression of the ODC gene, (b) retinoids affect the induction of ODC faster than any other parameter, (c) various levels of control of ODC activity are emerging rapidly, (d) ODC is an easy to measure parameter. In the case of the ODC induction by TPA, the following sequence of steps can be envisioned: TPA binds first to its receptor, this transmits a signal to the nucleus that results in the activation of the ODC gene and is followed by enhanced synthesis of ODC-messenger and enzyme. Theoretically retinoids can block ODC activity by interfering with any of these steps. Evidence is emerging that retinoids don't affect ODC activity at a post-translational level; our aim is to determine whether these compounds act on the level of gene expression and how.

As cell system we have chosen an established rat tracheal epithelial cell line 2C5 and primary rat tracheal cells as a model system since these cells are also used in studies concerned with the inhibition of neoplastic transformation by retinoids carried out in the carcinogenesis group.

The specific objectives are:

(1) To determine the effects of retinoic acid and TPA on the induction of ODC in 2C5 cells.

(2) To determine the mechanism of action of retinoids.

Phorbol esters induce ODC activity in rat tracheal cells. This induction is dependent on the incubation time, an optimum is reached at 4½ hrs. This induction is also dependent on the concentration of the phorbol ester, concentrations as low as 0.1 ng/ml are able to induce ODC activity. The increase in ODC activity is blocked by cycloheximide treatment and difluoromethylornithine. We have demonstrated the presence of TPA receptors. Analyses of Scatchard plots indicated the existence of two binding sites with high and low affinity. The structure-activity relationship of phorbol esters indicate that there is a good relationship between the biological activity and the binding to the receptor. These results suggest that the TPA-receptor may be involved in ODC induction. Retinoids inhibit the induction of ODC by phorbol esters. This action occurs rapidly: simultaneous addition of retinoids and TPA almost totally blocks induction. Binding proteins for retinoids have been identified in these cells. The structure-activity relationship for retinoids show a good correlation between the biological activity and the binding to the binding proteins and may suggest a role for the binding proteins in mediating this action of retinoids. Retinoids could reduce ODC activity at a post-translational level by activating transglutaminase, antizyme or another inhibitor. However, we have no evidence

that this occurs, in agreement with findings by others. Including retinoids in the in vitro assay mixture has also no effect on ODC activity.

Retinoids could interfere with the function of the TPA receptor. But before we could examine this possibility we had to demonstrate that the TPA receptor is indeed causal in ODC induction. The evidence that protein kinase C is the TPA receptor is strong. We examined the effects of diacylglycerol, the endogenous activator of protein kinase C, on the induction of ODC.

We have shown that several diacylglycerol derivatives can rapidly enhance ODC activity. The induction of ODC reaches an optimum at 3 hrs and is dose-dependent. Comparison of the structure-activity relationships show a good correlation between induction of ODC, inhibition of ^{3}H-PDBu binding to the TPA receptor and stimulation of protein kinase C activity. These results show that diacylglycerol mimics the action of TPA and suggests that protein kinase C is involved in the induction of ODC. Retinoids inhibit induction of ODC by diacylglycerol but do not affect ^{3}H-PDBu binding nor protein kinase C activity.

Retinoids inhibit the TPA-induced ODC activity in tracheal epithelial 2C5 cells. Retinoids might inhibit ODC activity at different levels such as the TPA receptor, the transmission of the signal to the nucleus, interaction with the chromatin, mRNA processing and at a post-translational level. Retinoids do not appear to affect ODC activity at a post-translational level. We have shown that activation of protein kinase C is causal in ODC induction. Retinoids do not inhibit the binding of TPA to its receptor nor affect protein kinase C activity. These results indicate that retinoids act at a level after the activation of protein kinase C.

To examine whether retinoids prevent the induction of ODC by inhibiting the expression of the ODC gene, we will measure the action of retinoids on the kinetics of $mRNA_{ODC}$ synthesis. If retinoids inhibit $mRNA_{ODC}$ synthesis two approaches can be taken to determine whether this occurs via a direct or indirect way. We can determine the interaction of the binding protein-retinoid complex with the chromatin to examine whether this complex interacts with specific sites at the chromatin. The involvement of protein kinase C precludes that phosphorylation reactions are part of the mechanism of ODC induction. Retinoids could affect the phosphorylation of specific substrates thereby inhibiting the signal to be transmitted to the nucleus. In this case one could assume that the expression of other genes linked to the phosphorylation of the same substrate are inhibited by retinoids as well. Examination of the effect of retinoids on TPA-induced phosphorylation of cellular proteins could address this possibility.

PUBLICATIONS

N/A

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25023-01 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Mechanism of Neoplastic Development in Airway Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

(Continuation of Project No. Z01 ES 25002-07 LPFT)

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Epithelial Carcinogenesis Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.1	2.1	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on mechanisms of neoplastic development in airway epithelial cells are being conducted with the aim to characterize pre-neoplastic epithelial cell variants on both the cellular and molecular level. By investigating important modulators of neoplastic progression, we hope to elucidate its cellular and biochemical basis.

An analysis of cell populations of transformed rat tracheal epithelial (RTE) cells indicates that transformed stem cells produce large numbers of cells not exhibiting transformed growth characteristics.

Studies on the role of the anchorage independent (ag^+) phenotype in neoplastic transformation of RTE cells indicates that ag^+ cell variants are being generated in preneoplastic RTE cell populations at a rate of about 10^{-4} per cell, per cell generation and that the ag^+ phenotype may not be an obligatory step in neoplastic transformation of RTE cells.

Chromosomal analyses of RTE cell transformants suggest that spontaneous and induced transformants have similar qualitative and quantitative chromosomal changes which might be important in the establishment of the EG-variant phenotype.

The tumor promoter TPA does not increase the frequency of the EG-variant phenotype but it may enhance the development of the ag^+ phenotype in some but not other preneoplastic RTE cell clones.

Retinoic acid inhibits RTE cell transformation at concentrations which are not inhibitory for growth of normal RTE cells. Whether RA inhibits the clonal expansion or the expression of RTE cell transformation is under study.

PROJECT DESCRIPTION

Many questions regarding the multistage model of carcinogenesis remain unresolved: the phenotypic characteristics of preneoplastic stages are ill defined; it is not known how many distinct preneoplastic stages can occur; the cellular and molecular mechanisms underlying progression and selection during neoplastic development are not understood. Over the last several years our laboratory has established an *in vitro* transformation system of rat tracheal epithelial (RTE) cells. This transformation system has many desirable features for studying qualitative as well as quantitative aspects of neoplastic development. Using this model it is possible to investigate preneoplastic epithelial cell variants developing *in vivo* as a result of carcinogen exposure or to study various phases of neoplastic cell transformation induced *in culture* by exposure of normal tracheal cells to carcinogens. In either case two preneoplastic stages are recognized which precede the development of neoplastic cell populations. The first is characterized by the appearance of so-called "enhanced growth (EG) variants" which give rise to transformed colonies, escape terminal differentiation and senescence and become immortal (acquiring cell line status, EG variant cell lines). The second preneoplastic stage is characterized by the appearance of cells which are anchorage independent (ag^+). Using the RTE cell transformation system the following main research objectives are currently being pursued:

1. Characterization of preneoplastic RTE cell variants. This work includes (a) efforts to define the cell populations produced by EG variants in terms of their proliferative capacity; (b) attempts to determine the role of the anchorage independent cell variant in the development of the neoplastic phenotype; and (c) attempts to define the preneoplastic disposition of early and late RTE cell variants in terms of cytogenetic abnormalities and molecular markers.
2. Determination of the responsiveness of different RTE cell variants to tumor promoters. We showed that the tumor promoter TPA markedly enhances the development of tracheal carcinomas *in vivo*. The purpose of the *in vitro* studies is to determine which preneoplastic cell variants are targets for tumor promoters.
3. Determination of the effects of antipromoters, such as retinoids, on different phases of neoplastic transformation of RTE cells.

Characterization of Preneoplastic RTE Cell Variants

Freshly-isolated RTE cells contain colony-forming cells which are able to produce epithelial colonies within 5 to 7 days after plating on 3T3 feeder layers. The proliferative capacity of these epithelial colony-forming units (CFU) is however limited, since after 12 to 14 days of culture their progeny ceases to proliferate. However when RTE cells are exposed to carcinogens, cell variants emerge which form continuously growing epithelial colonies, so-called enhanced growth (EG) variant colonies, under conditions which are non-permissive for growth of normal tracheal cells. The EG variant is the transformed stem cell of the RTE cell system. After repeated subculturing the EG variant becomes immortal, frequently anchorage independent and neoplastic; it is therefore considered to be preneoplastic.

A series of experiments were designed to study the cell populations comprising EG variant colonies. We asked the following questions: Do all the cells in transformed colonies exhibit the same replicative capacity (colony forming ability)? Do all the cells share the same transformed phenotype, i.e., the capacity to produce EG variant colonies? The results showed that the majority of cells in EG variant colonies had no demonstrable proliferative capacity and that only a minority of cells was able to produce EG variant colonies. Thus, we concluded that only a fraction of the offspring of transformed cells has transformed growth characteristics. However we also found that the proportion of cells in transformed colonies expressing the transformed phenotype continuously increases. Future studies will explore factors controlling the fractional size of the compartment of transformed stem cells.

Previous studies indicated that rat tracheal cells transformed by chemical carcinogens develop another growth related phenotypic change namely the capacity to grow in semi-solid medium (anchorage independence of growth = ag^+). This phenotype was commonly observed during late stages of neoplastic transformation and, as in other cell systems, appeared to either precede or coincide with the development of tumorigenic cell variants. To determine the precise role of the ag^+ phenotype in the progression of RTE cells from the preneoplastic to the neoplastic phenotype, a study was designed to determine the rate of spontaneous generation of ag^+ cell variants from ag^- preneoplastic cell variants. Another study was designed to examine the correlation between the ag^+ and the tumorigenic phenotypes.

Testing several EG variant cell lines which were anchorage dependent initially, it was found that the ag^+ variants arose spontaneously by a stochastic process at rates of $0.5X10^{-4}$ to $5.0X10^{-4}$ variants per cell per cell generation. This was established by fluctuation analysis. These rates of change to anchorage independence are similar to those reported in several fibroblast transformation systems. We also examined the frequency of induction of ag^+ cells in EG variant lines by exposure to MNNG. In these experiments we observed induced frequencies of 10^{-3} ag^+ variants per surviving cell which is at least one order of magnitude lower than that observed for induction of the EG variant in primary RTE cell cultures by the same carcinogen.

It is not known whether the ag^+ phenotype is an obligatory step in the evolution of neoplastic phenotypes or how closely it correlates with the neoplastic phenotype itself. Therefore tumorigenic RTE cells were injected into nude mice; the resulting tumors were dissociated and a variety of clones established from them were retested for tumorigenicity and for ag^+ growth. These studies, though not yet complete, did not show a close correlation between the ag^+ phenotype and tumorigenicity. However, recent findings suggest that marked differences may exist between various cell lines in terms of conditions optimal for expression of the ag^+ phenotype. Additional studies are therefore required before any final conclusions can be drawn.

To further characterize the EG variants, cytogenetic studies in RTE cell cultures before and after appearance of recognizable EG-variant colonies were performed. During the first 3 weeks of culture far more chromosomal abnormalities

(structural as well as numerical) were found in MNNG exposed than in control cultures (75% versus 8% at 3 weeks). At 5 weeks when transformed colonies are present in exposed and control cultures (though at a much lower incidence in the latter) roughly 50% of the metaphases scored in both groups showed abnormalities. Sixty percent of MNNG induced and spontaneous EG-variant colonies showed chromosomal abnormalities. The preliminary studies suggest that chromosome changes might be important in the development of EG-variants. The biological behavior of the EG-variant colonies not showing chromosomal changes (40%) will be examined and we will search for nonrandom chromosomal abnormalities in EG-variant colonies of different sizes and morphologies.

To investigate the role of oncogenes in the expression of different preneoplastic and neoplastic phenotypes in RTE cells, three approaches are being used: (1) Ag^- and ag^+ preneoplastic EG-variant cell lines are transfected with cloned oncogenes (v-ras, v-src and v-myc) and are subsequently assayed for presence or absence of the ag^+ and tum^+ phenotype. We thereby hope to determine whether preneoplastic RTE cells of different phenotypes are susceptible to transformation by specific oncogenes (or oncogene combinations). (2) To determine whether tum^+ RTE cells express activated oncogenes and which type(s), DNA is isolated from cloned tum^+ RTE cell lines, and applied to nontumorigenic NIH/3T3 mouse fibroblasts. Transformed foci are isolated and analyzed for rat gene sequences and attempts will be made to identify activated oncogenes. (3) In other studies RNA from preneoplastic and neoplastic RTE cell variants will be isolated and tested with oncogene probes such as ras, src and myc to search for expression of amplified oncogenes. These studies which have only recently been initiated indicate the suitability of techniques used routinely in NIH/3T3 transfection studies.

Promotion of Neoplastic Transformation of RTE Cells in Culture

Recently two provocative hypotheses have been advanced regarding mechanisms of action of tumor promoters. One postulates that promoters selectively induce terminal differentiation in nontransformed cells and stimulate replication of transformed cells. The other postulates that TPA causes chromosomal changes via some free radical mechanism, bringing about an additional qualitative change in initiated cells, leading to the emergence of the neoplastic phenotype.

Neoplastic transformation of RTE cells provides a particularly appropriate model to test these hypotheses. Promotion has been observed in whole tracheas *in vivo* as well as in organ culture and at least two preneoplastic stages have been identified in the cell culture model which is well suited for biochemical as well as quantitative cellular studies.

The experiments performed to date fall into two main categories: (a) assessment of growth stimulatory activity of various phorbol ester-related tumor promoters for RTE cells and (b) identification of preneoplastic cell variants susceptible to promotion. There is virtually no information on this latter subject in the literature except for a recent report indicating that TPA does *not* cause conversion of skin papillomas to carcinomas (while certain mutagens do). The studies with normal primary RTE cells showed that TPA (at concentrations of 10^{-9} M and above) caused marked stimulation of colony-forming efficiency (CFE). It

apparently induced "competence" to enter the cell cycle in cells with colony-forming potential and was most effective in this regard when given at the time of plating. When given at 48 hrs after plating 10^{-9}-10^{-7} M TPA had no effect. Other phorbol ester related tumor promoters as well as teleocidin also were potent stimulators of CFE.

Studies on the effect of TPA on transformation of RTE cells showed that it did not alter transformation frequency (i.e., frequency of EG variant colonies). Experiments on a later stage of RTE cell transformation, namely the transition of the ag^- immortal EG variant to the ag^+ variant, showed a heterogeneous response which may be typical of preneoplastic variants. In 1 out of 7 immortal ag^- cell lines TPA induced the ag^+ phenotype; out of 8 moderately ag^+ cell lines (CFE in agarose between 10^{-3}-10^{-2}) TPA enhanced the phenotype in 3 and suppressed it in 5 cell lines. The divergent responses of these 15 transformed RTE cell variants may be exemplary of the heterogeneity of responses of preneoplastic cells in general and may foreshadow the "tumor cell heterogeneity", which has received so much attention in recent years. Our experiments suggest that in a pool of preneoplastic cells exposed to tumor promoters, it may be only the rare cell which responds to the exposure with accelerated progression. Future experiments will determine whether TPA enhances the appearance of the neoplastic phenotype.

Inhibition of Transformation of RTE Cells by Retinoic Acid (RA)

Retinoids have been convincingly shown to be effective inhibitors of tumor induction in a number of animal models such as mammary gland, bladder and skin tumor models. In the animal studies the retinoid effects appeared to be due to inhibition of expression or clonal expansion of initiated or transformed cells. Various biochemical studies have suggested that retinoids may interfere with tumor promotion and indeed most of the tumor models in which retinoids are effective inhibitors have a tumor promotion component. Attempts to inhibit tumor induction in the airways of rats and hamsters have not been reproducibly successful. However such negative findings may simply be due to technical difficulties or limits of sensitivity of animal models. We therefore decided to reinvestigate the problem of inhibition of neoplastic development by retinoids using the *in vitro* RTE cell system.

Our initial objective was to determine whether RA inhibits transformation of RTE cell cultures. The studies showed that RA, when added to the cultures immediately after MNNG exposure, reduced the transformation frequency in a dose-dependent fashion. In these experiments RA was present in the cultures for the entire 5-week expression-selection period. Subsequent experiments showed that RA treatments as short as 1 week caused significant inhibition of transformation and that the start of treatment can be delayed for 2 weeks without loss of the inhibitory effect. This suggests that it might be primarily the clonal expansion of the transformants which was inhibited. Significantly, the RA concentrations used in these experiments caused little or no inhibition of either CFE or growth of normal RTE cells. Future studies will explore whether or not the observed RA effect is reversible, whether RA inhibits later stages of neoplastic transformation of RTE cells and what cellular and biochemical mechanisms are involved.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25024-01 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathogenesis of Early Pulmonary Lesions Induced by Inhaled Inorganic Particles

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Interstitial fibrotic lung disease is commonly found in humans exposed occupationally and environmentally to certain inorganic particles. We have established animal models to elucidate the initial cellular events associated with inhalation of the toxic dusts, asbestos and silica, and we have determined that a variety of inorganic particles, regardless of shape or concentration, deposits initially at bifurcations of alveolar ducts. It is at these sites of particle deposition that we have documented the earliest anatomic alterations induced by inhalation of asbestos fibers. These alterations at duct bifurcations include (1) phagocytosis of asbestos fibers by the alveolar epithelium, (2) translocation of fibers through the epithelium to capillary spaces and interstitial connective tissue, (3) phagocytosis of fibers by interstitial fibroblasts, (4) formation of interstitial intracellular microcalcifications, and (5) accumulation of alveolar and interstitial macrophages. Now, we report the following: (1) One month after a 1-hr exposure to chrysotile asbestos, the alveolar duct bifurcations exhibited normal epithelial cells, but there were significant increases in the numbers of interstitial fibroblasts and macrophages and in the amount of interstitial extracellular matrix, some of which was collagen. (2) Autoradiographic studies demonstrated that increased numbers of epithelial cells lining terminal bronchioles as well as epithelial and interstitial cells of alveolar duct bifurcations incorporated tritiated thymidine into DNA. (3) One month after the 1-hr exposure to asbestos, 19% of the fibers which had originally been deposited after inhalation was still present in the interstitium of the rats' lungs. Further studies are ongoing to establish the role of interstitial fibers and the mechanisms through which the fibers induce fibroblast proliferation and collagen production.

(Continuation of Project No. 25007-06 LPFT)

PROJECT DESCRIPTION

Inhalation of toxic inorganic particles such as asbestos and silica causes debilitating interstitial fibrotic lung disease in humans and experimental animals. Even though this has been known for decades, there is little information available on the basic cellular and biochemical mechanisms through which the particles injure lung cells and subsequently cause disease. To understand the pathogenesis of the pulmonary lesions, it is essential to establish the sequence of events associated with (1) initial particle depositiion patterns, (2) uptake and translocation of the particles among various cell types and anatomic compartments of the lung, (3) the physico-chemical changes and distribution of intrapulmonary particles and (4) the initial-cell tissue alterations which signal the development of the disease.

Our current objectives are designed to test three related hypotheses:

(1) We postulate that inhaled inorganic particles small enough to pass through the conducting airways are deposited initially at the bifurcations of alveolar ducts. Consequently, these anatomic regions are the sites where early cellular alterations progress to measurable interstitial lesions.

To establish initial deposition patterns of inhaled particles it is necessary to use brief (1-5 hr) inhalation periods, rapid intravascular fixation methods which do not disrupt particle location and dissection techniques which permit constant monitoring of precise microanatomic position. These objectives were accomplished by exposing white rats to asbestos in inhalation chambers for 1 hr. Immediately after exposure (4 min.±35 sec post-exposure), the animals were anesthetized with Nembutal and the lungs were vascularly perfused through the pulmonary artery with 1.0% saline and then a modified Karnovsky's fixative glutaraldehyde-1% paraformaldehyde). After 15 min of perfusion, the lungs were excised *in toto* and fixed overnight. Tissue blocks approximately 1 cm^3 then were dissected from right and left lungs and prepared for transmission and scanning electron microscopy (SEM). After critical point drying, the blocks were further dissected to reveal specific anatomic units of the distal lungs, i.e., terminal bronchioles (TB) and the associated alveolar ducts (AD) and alveolar spaces (AS). To address questions of structural alterations, the same fixation and tissue preparation techniques, were used on animals sacrificed after appropriate post-exposure periods, i.e., 12, 24, 48 hrs, and 1 month. Thus initial deposition patterns could be established by SEM, and consequent lesions could be studied by ultrastructural morphometry in tissue fixed and dissected simultaneously.

We found that chrysotile and crocidolite asbestos fibers, fiberglass, silica and Mt. St. Helens Ash particles are deposited initially at the bifurcations of alveolar ducts. Our data showed that regardless of shape or concentration, particles small enough to pass through the conducting airways were transported to the alveolar region of the lung by airflow significant enough to produce a consistent deposition pattern. Apparently, large numbers of particles were carried near the center of the flow and were intercepted by alveolar duct bifurcations intersecting the flow. This finding is significant for two reasons; first,

misconceptions regarding particle deposition in the alveolar regions have persisted since investigators have assumed that inhaled particles deposit along alveolar duct surfaces and within individual alveolar spaces. The common view is that air-flow velocity is very low past the terminal broncioles, and that particles are deposited randomly by diffusion. This proved not to be the case in rats and mice. Second, it is likely that the initial deposition pattern of inhaled toxic particles plays a role in the nature of the early cellular alterations caused by the particles. Knowledge of this deposition pattern has directed our morphologic studies to the alveolar duct bifurcations where we showed that the earliest cellular lesions of asbestosis are initiated. We showed that significant numbers of pulmonary macrophages accumulated at these sites of asbestos deposition within 48 hrs after a 1-hr exposure to chrysotile asbestos, whereas duct surfaces of sham-exposed rats were essentially devoid of macrophages. After quantifying this response by electron microscopy, we learned that over 90% of the bifurcations from exposed animals exhibited clusters of macrophages, whereas 85% of the sham-exposed bifurcations had no macrophages. The influx of macrophages was associated with a significantly increased bifurcation tissue area as determined by ultrastructural morphometry. This alteration persisted for at least one month after the 1-hr exposure. It was interesting to learn that two-thirds of the accumulated macrophages could be removed from alveolar duct bifurcations by bronchoalveolar lavage. These macrophages exhibited significant changes in surface morphology and in their phagocytic and chemotactic capacities. It seems reasonable to speculate that pulmonary macrophages of individuals exposed to asbestos for long periods in an occupational setting would exhibit similar alterations in form and function. Accumulation of macrophages and changes in cell form and function are likely to be amplified during chronic exposures and may be significant in leading to asbestos-induced lung disease.

Ongoing and future studies on the role of the macrophage in asbestos-induced lung disease are addressed in other concurrent annual reports. In this project we intend to pursue preliminary studies which have supported the hypothesis that intraepithelial actin-containing microfilaments play a role in the transport of inorganic particulates through the cytoplasm of alveolar epithelial cells. Future studies will employ microfilament and microtubule inhibitors in both in vivo and in vitro experiments. Knowledge of the mechanisms of particle transport is essential in understanding how asbestos fibers and silica crystals which impact on alveolar surfaces ultimately reach the lung interstitium where the fibrotic disease is manifested.

(2) We postulate that deposition of asbestos fibers at duct bifurcations results in injury of epithelial and interstitital cells manifested by increased incorporation of tritiated thymidine into DNA.

To determine whether or not pulmonary parenchymal cells are affected by asbestos deposition, asbestos-exposed and sham-exposed rats were injected IP with 2 μCi of tritiated thymidine (^{3}HThy)gm body weight 4 hrs before sacrifice by Nembutal injection. Animals were sacrificed after 24, 33, or 48 hrs recovery from exposure. Terminal bronchioles with adjacent alveolar duct bifurcations were selected from embedded lung tissue. Serial, .5 μm thick, sections as well as

thin sections from the selected anatomic units were cut and processed for autoradiography. The numbers of radioactive nuclei for each cell type were counted. Thin sections were used to identify cell types.

Results of our studies on asbestos-exposed rats injected with tritiated thymidine are preliminary at this point in time. However, it is quite clear that three hours of exposure to chrysotile asbestos causes increased numbers of epithelial cells lining terminal bronchioles to incorporate DNA into nuclei. In addition, there are significant increases in the number of ^{3}Hthy-positive cells at alveolar duct bifurcations. The precise nature of these cell types has not yet been determined, but it appears as though interstitial fibroblasts and epithelial cells are the types most commonly positive as determined by light microscopic autoradiography. Quantitative studies and electron microscopic identification of the first cells which are induced to replicate by asbestos fibers should lead to a more complete understanding of the cellular pathogenesis of asbestos-induced lesions. In addition, knowledge of which cells respond in vivo provides a sound basis for studying these cells in vitro.

Ongoing studies support the hypothesis that brief exposure to asbestos fibers causes injury of epithelial cells lining terminal airways and alveolar duct bifurcations and eventually interstitial fibroblasts. Future studies will clarify the precise cell types affected using light and electron microscopic autoradiography. These studies will be designed to establish whether or not the cell types which exhibit initial incorporation of DNA within the first 24-48 hrs after exposure are the progenitors of the progressive lesions we have measured one month post-exposure.

(3) We postulate that the progressive nature of asbestos-induced interstitial lung disease is due to a significant mass of inhaled dust which is retained in the lung interstitium for months (probably years) after cessation of exposure.

To determine the mass, numbers and dimensions of fibers retained in the lung at various times post-exposure, it was necessary to first establish these parameters immediately after a brief (1 hr) exposure to an asbestos aerosol. Rats were exposed in chambers as described earlier and sacrificed immediately after exposure, 24 hrs, 8 days, 2 weeks and 1 month post-exposure. Chrysotile asbestos was recovered from the lungs using hypochlorite digestion of the tissue. The digestate was then filtered through Nuclepore membranes (0.2 μ pore size) which provided a substrate upon which to visualize fibers in the SEM. After thorough rinsing, the filters were attached to carbon disks for fiber analysis. A minimum of 200 fibers per tissue sample was analyzed and the following parameters were measured directly for each time point: fiber length, diameter, and elemental content. Using data on these parameters in a newly developed formula, the mass of asbestos retained in the lung over time was calculated. The formula states that the mass of asbestos per rat lung in μg is equal to $N \cdot p \cdot l\pi r^2 \cdot w$, where:

N = the number of asbestos fibers/gm rat lung
p = density of chrysotile (2.55×10^{-6} μg/μm^3)
l = fiber length (microns)

r = fiber radius (microns)
w = weight of inflated fixed lungs (gms)

Then, knowing the concentrations of asbestos in the exposure chamber, the respiratory rate and tidal volume of the animal as well as exposure duration, the mass of inhaled asbestos can be calculated. Subsequently, the percentage of respirable fiber mass which is deposited can be calculated for the rats sacrificed immediately after exposure. Thus, using this value, the percentage of asbestos retained in the lung can be calculated at any time post-exposure using the formula described above.

Results showed that in rats exposed to chrysotile asbestos for 1 hr, there is a progessive decrease in fiber mass in the lungs over a 31-day period. The percentage of the original respirable mass which was deposited in the lungs was calculated to be 23% in rats sacrificed immediately after the 1-hr exposure. The most significant finding in this project is the determination that 19% of the originally deposited mass was still present in the lungs 31 days post-exposure. In addition, there was a statistically significant increase in fiber length and decrease in fiber diameter in the population of fibers isolated from the lungs 31 days after recovery as compared to earlier time intervals. Thus, we have shown that during the time interval in which asbestos fibers are transported from the air spaces into the interstitium or are taken up by alveolar macrophages, there is a progressive decrease in fiber number and mass, and significant changes are observed in dimensions of fibers retained in the lung. As previously reported, these fibers are initially deposited at alveolar duct bifurcations, are translocated through alveolar epithelial cells and subsequently are incorporated into the interstitium. These interstitial fibers are found in fibroblasts, and macrophages and extracellular connective tissue and are likely to play a major role in the progressive nature of asbestos-induced interstitial lung disease.

Ongoing studies have supported the hypothesis that after asbestos exposure, short fibers are cleared from the lung first, leaving a population of longer, thinner fibers in the interstitium. Future studies will address the issues of fiber distribution in various lobes and anatomic regions of the lung. It will be necessary to define the numbers of fibers and fiber dimensions in airspaces, macrophages, interstitial, and intravascular spaces to determine how these parameters change over time. In addition, the magnesium and silicon content of fibers in the various anatomic compartments will be monitored to determine whether or not the Mg:Si ration is altered *in vivo* and if so, does this correlate with cytotoxic events *in vivo* and *in vitro*.

PUBLICATIONS

Brody, A. R. and Roe, M. W.: Deposition pattern of inorganic particles at the alveolar level in the lungs of rats and mice. Amer. Rev. Resp. Dis. 128: 724-729, 1983.

Brody, A. R., Hill, L. H., and Adler, K. B.: Actin-containing microfilaments of pulmonary epithelial cells provide a mechanism for translocating asbestos to the interstitium. Chest 83: 11-12, 1983.

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Brody, A. R.: The early pathogenesis of asbestos-induced lung disease. Scan. Elect. Mic. I: 167-170, 1984.

Roggli, V. and Brody, A. R.: Changes in numbers and dimensions of chrysotile asbestos fibers in lungs of rats following short-term exposure. Exp. Lung Res., In press, 1984.

Oghiso, Y., Kagan, E., and Brody, A. R.: Intrapulmonary distribution of inhaled chrysotile and crocidolite asbestos: ultrastructural features. Brit. J. Exp. Pathol., In press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES-25025-01 LPFT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Asbestos Activation of Complement-Dependent Chemotactic Factors for Macrophages

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In earlier studies, we showed that pulmonary macrophages migrate to the sites where inhaled chrysotile asbestos fibers initially are deposited (i.e., surfaces of alveolar duct bifurcations). These macrophages form a major component of an early asbestos-induced interstitial lesion in rats. Thus, in order to establish the basic cellular mechanisms of asbestos-induced lung disease, it is essential to determine the chemical mediators which attract macrophages to these sites of fiber deposition. Chrysotile asbestos fibers used in vitro activate complement proteins in peripheral blood serum and in lavaged cell-free lung proteins. After brief inhalation of chrysotile asbestos, fluids lavaged from the lungs of exposed rats contain substantial chemotactic activity for macrophages compared to fluids from sham-exposed animals ($p < .01$). We hypothesize that this chemotactic activity is derived from complement activated by inhaled asbestos on alveolar surfaces. This contention is supported by the following observations: (1) Production in vitro of chemotactic activity by asbestos in serum or in lung lavageates was blocked by complement inhibitors. (2) Fractionation, by molecular sieve chromatography, of serum proteins and concentrated proteins lavaged from the lungs of asbestos-exposed rats showed that chemotactic activity was detected in the 14-18,000 MW range. This fractionation profile is similar to C5a, the chemotactic product of complement activation. In addition, (3) rats treated with cobra venom factor to deplete circulating complement as well as complement-deficient mice demonstrated significantly depressed macrophage accumulation at sites of asbestos deposition. Pulmonary macrophages are the cells which form the initial inflammatory response to asbestos inhalation. Our findings support the hypothesis that macrophages are attracted to the anatomic sites where inhaled asbestos fibers activate complement-derived chemotactic activity.

(Continuation of Project No. Z01 ES 25008-06 LPFT)

PROJECT DESCRIPTION

Pulmonary macrophages are believed to play a central role in the pathogenesis of particle-induced interstitial lung diseases. If this proves to be true, it will be essential to establish the mechanisms by which macrophages are recruited to sites of toxic particle deposition. Studies carried out to elucidate these mechanisms have recently demonstrated that inhaled chrysotile asbestos fibers which pass through the airways of rats and mice deposit preferentially on alveolar duct bifurcations. Within 48 hours after a 1-hour exposure, pulmonary macrophages migrated selectively to these sites of asbestos deposition and formed a component of an early lesion. This lesion was characterized by a two-fold increase in the mean tissue area of the bifurcation and persisted for at least one month after exposure to the dust. Our results are in agreement with morphologic studies in humans where the early characteristic lesions of asbestosis were manifested by macrophage accumulation at broncho-alveolar junctions.

Our ongoing studies address the central working hypothesis which states that inhaled asbestos fibers activate complement-derived chemotactic factors for macrophages on alveolar surfaces to produce pulmonary macrophage accumulation at sites of asbestos deposition.

Chrysotile asbestos fibers are known to activate serum complement and produce C5a, a potent chemotactic factor for neutrophils and macrophages. In addition, complement proteins, including C5, are normal constituents of distal lung fluids and are present on alveolar surfaces. Consequently, we have studied the production of complement-dependent chemotactic factors in vivo in serum and in proteins lavaged from the lungs of normal rats. The findings from these studies then are correlated with morphologic evidence of macrophage accumulation in the lungs of asbestos-exposed animals.

Male rats, 8-10 weeks of age, and congenic strains of C5 sufficient ($C5^+$) and deficient ($C5^-$) male mice, 4-6 weeks old, were utilized in these studies. Normal rats and rats treated with cobra venom factor, as well as the congenic strains of C5 sufficient and deficient mice were exposed for 3 hours to chrysotile asbestos in open cages contained within inhalation chambers. Control rats and mice were similarly sham-exposed to room air.

Immediately after, and 48 hours after a 3-hour exposure to the asbestos aerosol or room air, rats and mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After anesthesia the tracheas were exposed and clamped to prevent lung collapse. The trachea of each animal was cannulated and then perfused by gravity fluid flow of Karnovskys fixative at a pressure of 15 cm H_2O. Thirty minutes later, with the trachea still clamped to prevent collapse, the lungs were removed from the chest cavity and immersed in fresh fixative for 48 hours. Tissue blocks were processed for scanning electron microscopy (SEM) and dissected to reveal terminal bronchioles and corresponding alveolar ducts.

Complement Depletion and Monitoring

A group of 10 rats was decomplemented by an injection of Cobra venom factor (CVF) (50 units) into the tail vein one day before asbestos exposure. CVF (from Naja naja) was free of phospholipase A_2. Serum complement levels of rats treated with CVF were monitored using pulmonary macrophage chemotaxis to zymosan-activated rat serum as a bioassay for complement activation. Our results showed that serum complement levels were depressed by 85-95% within 4 hrs after injection.

Serum Complement Studies

Rat serum was separated from aggregated whole blood preparations. Fresh serum was incubated with zymosan (10 mg/ml) and Mt. St. Helen's ash particles, (25 mg/ml) as well as chrysotile asbestos (25 mg/ml), crocidolite asbestos (25 mg/ml) and fiberglass (25 mg/ml). Zymosan is a well known activator of complement and was used as a positive control. Ash particles from Mt. St. Helen's volcano did not activate serum complement and therefore served as a negative control. Additional negative controls were serum incubated without particles (37°C for 45 min) and heat-decomplemented sera (56°C for 30 min), which were then incubated with chrysotile asbestos fibers. Sera-particle mixtures were placed in conical centrifuge tubes and incubated in a water bath at 37°C for 45 min. Following incubation, the sera were decomplemented (56°C for 30 min).

Chemotaxis Studies

Pulmonary macrophage chemotaxis studies were carried out by using 100 ml of the test substance pipetted into the lower portion of a blind well chamber. A polycarbonate filter (pore size = 5 μm) was inserted between the lower and upper compartments. A concentration of 1.5×10^6 macrophages was pipetted into the upper compartment of the chamber. The chemotaxis assay was carried out for 3.5 hrs in an incubator (37°C), and the numbers of macrophages which had migrated from top to the opposite side of the polycarbonate filter were counted by light microscopy at 400X.

Fluid recovered from the lungs of asbestos and sham-exposed rats by phosphate buffered saline lavage was processed through 2 centrifugation steps, one (250 g for 10 min) to remove macrophages and an additional high speed spin (178,000 g for 60 min) to remove particulate lipid. The remaining solution was concentrated to a volume of 2-3 ml by dialysis against Aquacide and lyophilized. Concentrated lavaged proteins were resuspended in an RPMI 1640 buffer solution (containing 10 mM Hepes) at a concentration of 1.2 mg/ml and tested for chemotactic activity as described above.

In one set of experiments, lavaged proteins concentrated from normal unexposed animals were incubated in vitro with zymosan particles or chrysotile asbestos fibers in an attempt to produce complement activation of the cell-free lavaged fluids. In addition, several controls were used, i.e. in some tubes particles were incubated in the presence of 10 mM EDTA or 10 mM EGTA. In other tubes, lavaged proteins were decomplemented before incubation with chrysotile asbestos

fibers. Following incubation the supernate was removed and processed for chemotaxis studies as described.

Biochemical Investigations

Normal and zymosan-activated sera, as well as concentrated lavaged protein samples from sham and asbestos-exposed animals (30 mg protein in 2 ml) were chromatographed on a Sephadex G-100 column (1.5 cm X 55 cm) equilibrated with phosphate-buffered saline. Aliquots (0.1 ml) from each fraction were tested blindly for the capacity to produce a macrophage chemotactic response.

In our studies, we have attempted to elucidate the mechanism(s) by which pulmonary macrophages are attracted to sites of fiber deposition. Our results suggest that chrysotile asbestos fibers activate complement-dependent chemotactic activity in serum and lavaged proteins in vitro, and in vivo at alveolar duct bifurcations after asbestos inhalation. Complement activation by asbestos and zymosan generates a low molecular weight (14-18,000) chemotactic factor for macrophages. In addition, cell-free fluids lavaged from the lungs of unexposed animals were activated by asbestos in vitro, and this effect was inhibited by prior decomplementation or following incubation with EDTA, a Ca^{++} - Mg^{++} chelator. Yet, little inhibitory effect was observed following administration of EGTA, a Ca^{++} specific chelator. Since calcium and magnesium are essential cofactors for the classical and alternative complement pathways respectively, these findings suggest that chrysotile asbestos fibers activate complement via the alternative pathway in serum and lung fluids to produce complement-dependent chemotactic factors.

The inhalation studies also supported the hypotheses that complement, particularly C5, plays a role in stimulating the recruitment of pulmonary macrophages to sites of asbestos deposition. Congenic strains of C5 sufficient ($C5^{+}$) and deficient ($C5^{-}$) mice as well as normal and cobra venom factor (CVF)-treated rats were exposed to chrysotile asbestos. The numbers of macrophages at sites of fiber deposition were significantly depressed in the $C5^{-}$ mice and CVF-treated rats. This result was not due to intrinsic functional impairment of the $C5^{-}$ and CVF-treated macrophages, since these cells demonstrated a normal chemotactic response to zymosan-activated sera.

We have shown that pulmonary macrophage chemotaxis is enhanced following in vitro activation of complement in rat serum and in vivo in the lungs of asbestos-exposed animals. Complement proteins are normal constituents of distal lung fluids and are present in biologically active concentrations in lavage fluid recovered from normal animals. Therefore it seems reasonable to suggest that inhaled asbestos fibers activate complement proteins at sites of fiber deposition. On the other hand, the chemotactic response of pulmonary macrophages to sites of initial particle deposition may not be unique to chrysotile asbestos. A variety of particles, including crocidolite asbestos, cereal grain dust, zymosan, and fly ash are known to activate serum complement. Therefore, to establish the significance of our findings it will be important to identify the deposition pattern of a variety of inhaled particulates and to determine whether the initial inflammatory response coincides with macrophage migration to

sites of particle deposition as demonstrated here after exposure to chrysotile asbestos fibers.

We have not observed increased numbers of polymorphonuclear leukocytes (PMN) in the lungs or lavage fluids of rats briefly exposed to inhaled asbestos. Likewise, no significant increase in PMN was found in ultrastructural morphometric studies of chronically exposed rats. However, neutrophils are known to respond dramatically to complement-derived chemotactic factors such as C5a. Yet, macrophages are the cell-types which form the initial inflammatory response following inhalation of chrysotile asbestos. This apparent discrepancy could be explained by consideration of the logistics of the activation response. Conceivably, the small quantities of chemotactic factor (postulated to be C5a) generated on alveolar surfaces are unable to diffuse through epithelial barriers into the vasculature where they could be detected by circulating neutrophils and monocytes. As a result, it appears that chemotactic signals induced by the inhaled fibers are only expressed locally on alveolar surfaces where macrophages normally reside.

To our knowledge, we have presented the first demonstration of chemotactic factor generation and a corresponding macrophage inflammatory response on alveolar surfaces. We conclude that the asbestos-activated complement-derived chemotactic factor is C5a. This may be significant in the pathogenesis of lung disease since it has recently been shown that complement-associated chemotactic factors stimulate macrophages to secrete lysosomal hydrolases and toxic oxygen radicals. Secretion of these and other products may cause cell injury and facilitate the development of interstitial fibrogenesis.

The following experiments will be carried out to further test our hypothesis of complement-dependent macrophage accumulation:

(1) Establish time course of factor appearance and disappearance in correlation with dust exposure and macrophage accumulation.

(2) Correlate capacity of a dust to activate complement in vitro with its ability to attract macrophages in vivo after inhalation.

(3) Study other potential chemotactic factors such as macrophage and epithelial products which could be produced in the experimental system.

Related experiments are designed to study the role of macrophages in mediating the distribution of interstitial particles and in modulating interstitial fibrogenesis.

(4) Quantify asbestos distribution in various anatomic compartments of the lung in normal rats and in animals with decreased macrophage accumulation consequent to complement depletion.

(5) Correlate complement depletion (thus diminution of macrophage accumulation) with development of the interstitial lesion.

PUBLICATIONS

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Warheit, D. B., Hill, L. H., and Chang, L. Y., Crapo, J. D., and Brody, A. R.: Pulmonary macrophage accumulation and asbestos-induced lesions at sites of fiber deposition. Amer. Rev. Resp. Dis. 129: 301-312, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interactions of Inorganic Particles with Pulmonary Cell Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*
(Continuation of Project No. Z01 ES 25009-05 LPFT)

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SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Inhaled particles such as asbestos and silica are toxic to pulmonary cells. In recent studies on mechanisms of membrane injury, we have shown that chrysotile asbestos causes damage to erythrocyte membranes through binding to terminal sialic acid (SA) residues. The hemolytic events involved (1) binding of the positively-charged chrysotile fibers to negatively-charged SA groups, (2) rapid (within 5 min) distortion of the cells, (3) redistribution of SA groups, and (4) alterations of intracellular Na^+, K^+ ratios. Negatively-charged crocidolite asbestos bound to and distorted red cells but had no effect on SA groups or ion flux. To establish whether or not similar mechanisms of membrane injury play a role in particle-induced toxicity of pulmonary cells, we have extended our studies to pulmonary macrophages. Our hypothesis is that non-specific (i.e., non-receptor mediated) binding and subsequent uptake of positively-charged particles are mediated by negatively-charged cell surface sialic acid groups. In support of this hypothesis we have shown the following: (1) Wheat germ agglutinin (WGA), a lectin which binds to sialic acid, is distributed evenly across macrophage surfaces. (2) Positively-charged carbonyl iron (Fe) spheres and chrysotile asbestos fibers bind to macrophage membranes at 4° C and the binding is blocked by a dose-dependent pretreatment of the cells with WGA. Other lectins such as Ricin and ConA do not inhibit binding at comparable doses. (3) Removal of cell-surface glycoproteins with neuraminidase and periodate inhibit binding of the positively-charged particles. (4) Fe-spheres normally are readily phagocytized by macrophages at 37° C. In the presence of WGA, over 90% of the phagocytic activity is blocked, but other lectins have no effect. These studies support our hypothesis that charged surface sialic acid groups play a role in particle binding and phagocytosis. Further studies are ongoing to substantiate our findings using negatively-charged particles and membrane markers of receptor turnover.

PROJECT DESCRIPTION

Inhaled inorganic particles are phagocytized by a variety of pulmonary cells including macrophages, epithelial and endothelial cells and fibroblasts. Some particles such as asbestos and silica are toxic and induce cell death. The mechanisms through which the particles bind to cell membranes, are phagocytized and ultimately cause cytotoxicity are largely unknown. To address these issues, which are fundamental in understanding the pathogenesis of particle-induced lung disease, we are studying the mechanisms of particle binding to macrophage membranes and subsequent phagocytic events. Work accomplished to date supports the hypothesis that positively-charged particles bind initially to negatively-charged sialic acid groups on the surfaces of macrophage membranes. Whether or not this event is an essential element in subsequent phagocytosis and cell death remains to be determined.

Our current hypothesis states that the toxicity of chrysotile asbestos is mediated through its capacity to bind to sialic acid groups on cell membranes. To address this hypothesis, the following objectives are being pursued:

(1) to establish the distribution of sialic acid (SA) residues on macrophage membranes before, during and after phagocytosis of toxic and non-toxic particulates.

(2) to determine whether or not positively-charged particles such as iron spheres (Fe) and chrysotile asbestos bind to the SA groups.

(3) to test the effects of various lectins on rate and efficiency of phagocytosis by macrophages exposed to various particles.

To determine the mechanisms through which particles bind to macrophage membranes, cells were recovered from the lungs of normal rats by saline lavage. Macrophages were placed in RPMI medium without serum and allowed to attach to Thermanox cover slips for 45 min, at 37° C in a CO_2 incubator. The majority of the cells attach and spread out on the plastic substrate and have been shown to avidly phagocytize large numbers of iron spheres or asbestos fibers within 45 min after treatment.

The distribution of sialic acid (SA) groups on macrophage membranes was determined by conjugating the lectin wheat germ agglutinin (WGA) to colloidal gold spheres (Au). Since WGA binds to SA and Au spheres can be visualized under the electron microscope, the Au-WGA provides a label which can be readily quantified. Other lectins, such as ricin which binds to beta-galactose and CON-A which binds to mannose, were used as controls. To remove SA groups from the cell membranes, neuraminidase was used to specifically cleave the SA residues and periodate to remove surface glycoproteins.

To separate initial binding events from phagocytosis, a series of experiments was carried out on ice at 4° C. No phagocytosis of particles or turnover of SA groups could be detected under these conditions. Thus, in one group of experiments, particles such as Fe and asbestos were added to attached cells before or after addition of the various lectins. In other experiments, phagocytosis ensued after the cells were placed in a CO_2 incubator at 37° C.

After the various treatments, cells on plastic substrate were fixed, dehydrated and critical point dried for electron microscopy.

Wheat germ agglutinin bound to colloidal gold spheres was found to be distributed evenly over the macrophage membranes, on both ruffled and smooth cells. Binding of Au-WGA spheres was inhibited by pretreatment of the cells with WGA alone.

Forty-five minutes after addition of positively-charged iron spheres to the culture medium, over 70% of the macrophages contained large numbers of particles. This percentage did not increase after 4 hrs of culture. When the iron particles were added to the cultures at 4° C, no measurable phagocytosis took place and over 90% of the cells had particles bound to the cell surfaces. Vigorous washing did not remove spheres bound to macrophage membranes, but did clear the plastic substrate of most unbound iron particles. When the macrophages were pretreated with WGA before addition of iron spheres at 4° C, particle binding was inhibited in a dose-dependent fashion, e.g., .1 mg/ml of WGA inhibited binding by 90%. Similarly, pretreatment of cells with neuraminidase and periodate significantly diminished particle binding to macrophage membranes. The same experiments were carried out using chrysotile asbestos as the positively-charged particle, and the results were essentially identical.

As further tests for the role of SA in particle binding, lectins which bind to other cell surface sugars were used in the experiments described above. Ricin which binds to beta-galactose, ConA which binds to mannose and bovine serum albumin as a non-specific protein all had no measurable effect on particle binding through a wide range of concentrations.

The effect of WGA and the other lectins on phagocytosis was studied. WGA, whether added before, during, or after particle binding clearly inhibited phagocytosis of iron spheres. The other lectins had no measurable effect when the numbers of bound and internalized particles were determined over time.

These results support our hypothesis that positively-charged particles bind to negatively-charged sialic acid groups on macrophage membranes. This mechanism of initial binding seems essential in the process of non-receptor mediated, so-called "non-specific" phagocytosis. We propose that these events are integral to the toxicity manifested by a variety of inorganic particulates. Future studies will be directed toward addressing the mechanisms of toxicity.

Inasmuch as particle phagocytosis and consequent cytotoxic reactions are likely to play a central role in the pathogenesis of particle-induced lung disease, we plan to carry out the following experiments:

(1) Determine the turnover rate of labeled sialic acid on macrophage membranes and whether or not labeled sites maintain normal distribution subsequent to endocytosis of toxic and non-toxic particulates.

(2) Study the ultrastructural associations between phagolysosomal membranes and toxic or non-toxic particles since one working hypothesis states that phagocytized toxic particles such as asbestos cause disruption of phagolysosomal membranes through mechanisms similar to those expressed on cell surfaces.

(3) Extend our studies of binding and phagocytosis to negatively-charged particles such as silica, fiberglass and crocidolite asbestos.

PUBLICATIONS

Brody, A. R., George, G., and Hill, L. H.: Interactions of chrysotile and crocidolite asbestos with red blood cell membranes: Chrysotile binds to sialic acid. Lab. Invest. 49: 468-475, 1983.

Brody, A. R., and Hill, L. H.: Interactions of chrysotile asbestos with erythrocyte membranes. In Brown, R. C. et al. (Eds.), The In vitro Effects of Mineral Dusts, Environ. Health Perspect. 51: 85-89, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25027-01 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Identification and Characterization of Materials Secreted by Pulmonary Clara Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. E. R. Hook	Research Chemist	LPFT, NIEHS
Others:	S. E. Patton	Postdoctoral Fellow	LPFT, NIEHS
	A. M. Jetten	Senior Staff Fellow	LPFT, NIEHS
	P. Nettesheim	Chief	LPFT, NIEHS
	L. B. Gilmore	Biologist	LPFT, NIEHS
	L. A. Dethloff	Biological Lab. Tech.	LPFT, NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Biochemical Pathology Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The functions of the nonciliated bronchiolar epithelial cell (Clara cell) of the lungs are not known. Numerous morphological investigations in many species have led to the hypothesis that the Clara cell is secretory although the nature of those secretions and their function in the airways are not known. The objectives of this research are to eludicate the secretory nature of the Clara cell, identify and characterize those secretions and determine their extracellular functions. We have developed a model system for the study of Clara cell functions and metabolism using cells isolated from the lungs of rabbits. Cells were dispersed from lung tissue using a pancreatic protease introduced via the trachea. These dispersed cells contained approximately 8% Clara cells. The Clara cells were enriched to about 40% by centrifugation on a continuous density Percoll gradient. The Clara cells were then subjected to centrifugal elutriation, resulting in further purification (Clara cells 75-85% pure). These cells were added to collagen I coated culture dishes and incubated overnight in Ham's F12 medium. The attached cells consisted of at least 90% Clara cells. The isolated Clara cells contained abundant endoplasmic reticula and osmiophilic cytoplasmic granules typical of Clara cells *in vivo*. These features were maintained when the cells were incubated for 24 hours. Incubation of the isolated cells with ^{35}S-methionine for 4 hours resulted in the radiolabelling of many intracellular proteins some of which were released into the incubation medium. The major radiolabelled protein present in the medium had a molecular weight of about 10,000 daltons as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions. Clara cells could be maintained in *in vitro* culture for periods up to 2 weeks in Ham's F12 medium supplemented with insulin, transferrin, EGF, hydrocortisone, bovine hypothalamus extract and antibiotics. These studies indicate that the isolated Clara cells may be an appropriate model for the study of Clara cell function and metabolism.

PROJECT DESCRIPTION

The pulmonary functions of the nonciliated bronchiolar epithelial cells (Clara cells) are obscure. Recent investigations indicate that the cell is a target for a variety of toxic chemicals although the significance of this sensitivity to toxicants in relation to pulmonary functions in general has not been determined. Numerous morphological investigations in a variety of species have led to the hypothesis that the cell is secretory although the nature and functions of those secretions are not known. Our immediate objectives are to establish a model system wherein this hypothesis, that Clara cells are secretory, may be tested. If the hypothesis is true then the nature of the materials secreted will be investigated and characterized according to chemical composition and physical properties.

Many cell types exist in the airway epithelium and the study of Clara cells in that epithelium is currently unfeasible on technical grounds. Materials released by cells of the epithelium cannot be ascribed to any particular cell type with certainty. Our approach has been to develop methods for the isolation of Clara cells from the airway epithelium of the lungs of rabbits in highly purified form and with a high degree of viability. Highly purified populations of Clara cells are essential for the unequivocal identification of materials secreted by these cells. With appropriate incubation conditions these isolated Clara cells will be established as a model system for the study of Clara cell functions and metabolism. "Appropriate" conditions will require maintenance of the general morphological appearance of the Clara cell, maintenance of cellular markers such as formalin insensitive-nitro blue tetrazolium reductase and cytochrome P450-linked hydroxylases, and the secretion of materials that correspond with components of the pulmonary extracellular lining.

The literature suggests that if the Clara cell is secretory then two of the most likely candidates in these secretions are proteins and/or phospholipids. Clara cells in our model system should incorporate radiolabelled precursors into protein and/or phospholipids with subsequent release into the incubation medium.

We have developed a method for the isolation of Clara cells from the lungs with purity greater than 90% and viability of at least 95%. This method is a modification of the procedure developed by Devereux and Fouts using pancreatic proteases for the dispersal of lung cells. Upon dispersal of the cells from the lungs, Clara cells accounted for approximately 8% of the total cells. Removal of macrophages and leucocytes from these cell mixtures was achieved using continuous density Percoll gradients resulting in purification of Clara cells to about 40%. Clara cells were further purified (75-85%) by using centrifugal elutriation. Identifiable cells such as Type II cells, ciliated cells, goblet cells, leucocytes and macrophages together accounted for less than 10% of the total cells. Overnight incubation of the cells in collagen-coated dishes with Ham's F12 medium resulted in the attachment of Clara cells (40-50%) to the collagen matrix. The attached cells consisted of at least 90% Clara cells according to their appearance under the electron microscope and their ability to reduce nitro blue tetrazolium. The cells contained abundant smooth endoplasmic reticula and osmiophilic "secretory" granules, features characteristic of Clara cells.

Our first specific objective was to achieve an overall view of proteins synthesized using the isolated Clara cells and to identify proteins released by those cells into the incubation medium. In developing isolated cells as a model system for the study of Clara cell metabolism and function, a recovery period was considered necessary; however, lengthy recovery periods could also lead to alterations in cellular function and metabolism as the cells adapt to their new environment. Freshly-isolated Clara cells and Clara cells incubated for 18 hours in Ham's F12 medium were investigated; which of these two conditions was best suited for the study of Clara cells was not known and consequently we chose to study both. Incubation of the cells with ^{35}S-methionine or a mixture of ^{14}C-labelled amino acids resulted in the biosynthesis of numerous radiolabelled intracellular proteins some of which were also released into the incubation medium. In the presence of ^{35}S-methionine three conspicuous proteins with molecular weights of 50,000, 40,000, and 10,000 daltons were detected in the medium using SDS-PAGE in the presence of reducing agents followed by radioautography. These three proteins remained conspicuous after incubation of the cells for 18 hours with ^{35}S-methionine. In the 4-hour incubation the 50,000, 40,000, and 10,000 proteins contained 16, 6.5, and 42%, respectively, of the total extracellular radiolabel associated with proteins. Because of the large proportion of label incorporation into the 10,000 MW protein, its presence in the medium may be highly significant insofar as secretion by the Clara cell is concerned. This low molecular weight protein was also present in the Clara cells where it accounted for approximately 40% of the total radiolabel incorporated from the ^{14}C-amino acid mixture during the 4-hour incubation. Proteins synthesized and released by Clara cells will be further characterized according to their existence as glycoproteins or as subunits of larger proteins.

Our second objective is to determine how the protein synthesizing activity of isolated Clara cells differs from that of other pulmonary cells. Type II cells, alveolar macrophages, and tracheal epithelial cells will be incubated under conditions similar to those used for Clara cell investigations and proteins synthesized and released will be identified using SDS-PAGE followed by radioautography. Preliminary data indicates that the 10,000 dalton protein synthesized and released from Clara cells may be also synthesized by isolated Type II cells but not by alveolar macrophages.

Our third objective and that which will receive major attention in the subsequent year is to identify which of the proteins present in the pulmonary extracellular lining are secreted by Clara cells. For this purpose antisera against rabbit Clara cells will be prepared in goats. Non-Clara cell specific antibodies will be absorbed from the antisera using rabbit liver homogenates. These antisera will enable us to identify which proteins present in the pulmonary extracellular lining are Clara cell specific. These antisera will also allow the subcellular localization of the antigen to be established by using the immunoperoxidase histochemical staining procedure.

PUBLICATIONS

N/A

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES-25028-01 LPFT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Basis for Cellular Changes in Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. C. Barrett	Research Chemist	LPFT, NIEHS
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	M. Koi	Visiting Fellow	LPFT, NIEHS
	D. Thomassen	Postdoctoral Fellow	LPFT, NIEHS
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COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Pulmonary Function and Toxicology

SECTION
Environmental Carcinogenesis Group

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Neoplastic development of Syrian hamster embryo (SHE) cells is a multistep process. We have examined the influence of chemical carcinogens and the role of cellular and viral oncogenes in this process. We have compared the susceptibilities of normal and carcinogen-induced preneoplastic SHE cells to neoplastic transformation following transfection by the calcium phosphate precipitate techniques with plasmids of genomic clones of four oncogenic viruses: polyoma virus, Harvey murine sarcoma virus (Ha-MSV), Rous sarcoma virus (RSV) and MC29 virus (pSVv-myc). Normal SHE cells transfected with polyoma virus DNA formed progressively growing tumors of hamster origin within 3-4 weeks when injected into nude mice. In contrast, SHE cells treated with Ha-MSV DNA remained nontumorigenic. SHE cells treated with RSV DNA formed one tumor (one of six sites) with a latency period of 15 weeks. SHE cells transfected with either Ha-MSV DNA and pSVv-myc DNA or RSV DNA and pSVv-myc DNA formed tumors with short latency periods. Polyoma virus DNA, Ha-MSV DNA or RSV DNA could individually neoplastically transform preneoplastic SHE cells which were immortalized following treatment with the carcinogen diethylstilbestrol. These results suggest that multiple changes or activated oncogenes are required for the neoplastic transformation of SHE cells. To determine if normal cellular factors or genes can regulate the phenotypic expression of tumorigenicity and/or oncogenes, cell-cell hybrids between chemically transformed SHE cells and either normal or preneoplastic SHE cells were prepared. Our results indicate that anchorage independence, which is a good marker for tumorigenicity of these cells, is suppressed in hybrids between tumorigenic and normal cells and in hybrids between tumorigenic and most but not all preneoplastic cells. This suggests that this suppressive ability may be lost during neoplastic progression and represents one step in this process.

PROJECT DESCRIPTION

Neoplastic development of Syrian hamster embryo (SHE) cells in culture is a multistep process. Following exposure to chemical carcinogens, normal, diploid SHE cells give rise to altered cells which are nontumorigenic but have an increased propensity to become neoplastic. These intermediate cells, termed preneoplastic cells, can be isolated and cloned and exhibit a number of phenotypic differences from SHE cells, including an abnormal karyotype, altered morphology and an indefinite lifespan or immortality in culture. Cellular genes, homologous to retroviral oncogenes, have been identified and it has been proposed that they play a major role in the neoplastic development of cells in vitro and in vivo. To understand the nature of carcinogen-induced events in the neoplastic progression of SHE cells in culture and the role of oncogenes in this process, and to further characterize normal and preneoplastic cells, we have attempted to achieve the following objectives:

1. To determine the role of expression or mutations of oncogenes in the multistep process of neoplastic development of Syrian hamster embryo cells in culture.
2. To determine the effects of chemical carcinogens on the expression and/or mutation of relevant oncogenes during neoplastic progression.
3. To identify factors and genes which regulate the expression of tumorigenicity and/or oncogenes.

We have compared the susceptibilities of normal and preneoplastic SHE cells to neoplastic transformation following transfection by the calcium phosphate percipitate technique with plasmids of genomic clones of four oncogenic viruses: polyoma virus, Harvey murine sacroma virus (HaMSV), Rous sarcoma virus (RSV) and MC29 virus (pSVv-myc). Normal SHE cells which are stably nontumorigenic in nude mice are competent to take up and express exogenous DNA as demonstrated by transfection experiments with pSV2-neo DNA and certain viral DNAs. Transfection of the cells with 1 μg of polyoma virus DNA, which contains three oncogenes, and then injection of 4×10^6 transfected cells into nude mice resulted in progressively growing tumors of hamster origin within 3-4 weeks. In contrast, SHE cells treated with 5 μg of HaMSV DNA remained nontumorigenic. The HaMSV DNA treated cells grew in soft agar at a low frequency, but were morphologically normal and did not escape cellular senescence. Treatment of SHE cells with HaMSV DNA and pSVv-myc DNA (1 μg each) and injection into nude mice resulted in tumors within 3-5 weeks after injection. SHE cells treated with 5 μg of RSV DNA formed one tumor (out of 6 sites) in nude mice with a long latency period of 15 weeks. SHE cells treated with RSV and pSVv-myc DNAs formed tumors within 3-7 weeks. These results suggest that multiple changes or activated oncogenes are required for the neoplastic transformation of SHE cells and that v-src may be more efficient or pleiotropic than v-Ha-ras oncogene in affecting one or more of these changes.

A preneoplastic cell line, DES-4, isolated after treatment of SHE cells with the human carcinogen diethylstilbestrol, was chosen for comparative studies. These immortalized cells are nontumorigenic and excellent recipients for exogenous DNA. In contrast to SHE cells, DES-4 cells were highly susceptible to neoplastic transformation following transfection with either HaMSV DNA, RSV DNA,

or polyoma DNA. Treatment of DES-4 cells with as little as 10 ng of any of these DNAs resulted in a 100% tumor incidence in nude mice. The cells remained nontumorigenic after treatment with pSVv-myc DNA, carrier DNA alone, or plasmid DNA lacking the HaMSV oncogene (v-Ha-ras) or RSV oncogene (v-src).

To further investigate the role of the oncogenes in the neoplastic transformation of DES-4 cells, clones of DES-4 cells cotransfected with pSV2-neo and HaMSV DNAs were isolated by antibiotic resistance and characterized. There was a good correlation between tumorigenicity and expression of v-Ha-ras expression in the clones; however, the clones were highly variable in terms of their latency periods in vivo and anchorage-independent growth. Neither of these two parameters correlated with v-Ha-ras RNA expression. Cell lines derived from tumors had short latency periods in vivo, were highly anchorage independent, and had high levels of v-Ha-ras expression. These results suggest that v-Ha-ras expression is necessary but not sufficient for the tumorigenicity of DES-4 cells and that additional changes in the cells are required. Thus, our findings indicate that in spite of its oncogenic potential in vivo, the oncogene of HaMSV alone is insufficient to cause neoplastic transformation of normal or preneoplastic cells in culture. Two steps are required for preneoplastic DES-4 cells to become tumorigenic suggesting that at least three steps may be required for the neoplastic progression of normal SHE under certain conditions.

In contrast to the results with HaMSV, DES-4 cells cotransfected with RSV DNA and pSV2-neo DNA and selected for neo^R appear to acquire neoplastic potential as a single step. All clones with an intact RSV genome and expressing v-src RNA were highly tumorigenic with short latency periods (<3 weeks) and highly anchorage independent. These results further support our hypothesis that v-src is more efficient than v-Ha-ras in inducing neoplastic transformation and indicate that the number of steps observed in neoplastic development may depend on the oncogene activated.

Future studies will be directed at determining the cooperation of oncogenes in the neoplastic transformation of SHE cells, examining the expression of cellular oncogenes in the neoplastic progression of the cells following carcinogen exposure and relating carcinogen action to oncogene activation.

To determine if normal cellular factors or genes can regulate the phenotypic expression of tumorigenicity and/or oncogenes, cell-cell hydrids between chemically transformed SHE cells and either normal or preneoplastic SHE cells were prepared by fusing cells with polyethyleneglycol and isolating hybrid cells by selection for biochemical markers. Our results to date indicate that anchorage independence which is a good marker for tumorigenicity of these cells is suppressed in hybrids between tumorigenic and normal cells and in hybrids between tumorigenic and most but not all preneoplastic cells. This suggests that this suppressive ability may be lost during neoplastic progression and represents one step in this process. Further studies to confirm this conclusion and to identify these genes are in progress.

PUBLICATIONS

Gilmer, T. M., Thomassen, D. G., Annab, L. A., Oshimura, M., and Barrett, J. C.: Influence of chemical carcinogens and viral oncogenes on the neoplastic progression of Syrian hamster embryo cells. In Bishop, J. M., Greaves, M., and Rowley, J. D. (Eds.): Genes and Cancer, UCLA Symp. on Mole. and Cell. Biol., New Series, Vol. 17, Alan R. Liss, Inc., New York, 1984.

OAK RIDGE NATIONAL LABOBATORY, OAK RIDGE, TENNESSEE 37831
(222Y01-ES-80041)

TITLE: Study of Antigenic Markers in Developing Epithelial Neoplasia

CONTRACTOR'S PROJECT DIRECTOR: S. J. Kennel, Ph.D.

PROJECT OFFICER (NIEHS): Paul Nettesheim, M.D., Chief, LPFT

DATE CONTRACT INITIATED: October 1, 1983

TERMINAL YEAR FUNDING

PROJECT DESCRIPTION

During cell transformation and the development of tumorigenic properties, several biological stages can be identified by the cells' behavior in tissue culture. Changes in DNA content and cell surface antigen expression characteristic of particular stages of neoplastic transformation of rat tracheal epithelial cells have been quantitated. Thus it is possible to identify and quantitate subpopulations of cells which have a defined biological property in mixed cell cultures and study the appearance and growth interactions of these cell types during neoplastic transformation.

The objectives of this project are to quantitate rat tracheal epithelial (RTE) cell populations with specific biological properties and study their relative growth rates during the emergence of neoplastic cells from a preneoplastic population. Specifically, we want to determine if the conversion of 2-10-1 preneoplastic to neoplastic cells occurs through a single clonal event followed by growth domination of that cell type or if many or all preneoplastic cells in the culture possess the ability (preprogrammed) to convert to a similar neoplastic phenotype.

We would also test the MoAb's we have produced to RTE cells for their ability to bind to malignant and non-malignant cultures of rat embryo fibroblasts transformed with Rauscher leukemia virus.

This study involves changes in phenotypic expression of antigenic markers on rat tracheal epithelial cells (RTE) as they progress from preneoplastic (nontumorigenic) to neoplastic (tumorigenic) populations in culture. Carninogen-altered RTE cell lines have been established from carcinogen exposed F344 rat tracheas. Many of these continuous cell lines are not capable of forming tumors when inoculated into compatible animals at early cell passages but acquire this ability upon serial passage in vitro. These carcinogen-altered cell lines are phenotypically distinguishable from normal populations by the appearance of new cell surface antigens (Braslawsky et al., 1982a) and altered DNA contents (Braslawksy et al., 1982b).

In previous progress on this project, we have developed MoAb that bind to tumorigenic cultures of RTE cells. Serological recognition of cell surface antigens are divided into two categories: those which assess average antigen expression of the cell population and those which measure antigen expression and density of individual cells within the population. The radiolabeled antibody binding test (ABT) is of the first type. This test is used to quantitate the

amount of tumor antigen on preneoplastic and neoplastic cell populations. The fluorescent antibody test analyzed by flow-cytometric methods is of the second type, which analyzes cell populations for fluorescent yield per cell as well as enumerates the percentage of antigen positive oells. These results are correlated with the relative DNA content of individual cell types. Relative DNA content is determined by staining cells with DNA specific fluorescent dyes and quantitating the florescence by flow-cytometric methods. RTE cells from non-carcinogen age-matched animals serve as control populations and are used to establish baseline values for comparison of results obtained using caroinogen-exposed RTE. These cells, reagents and techniques provide us with a unique opportunity to identify and quantitate individual cell phenotypes in mixed cell populations as they develop neoplastic potential in culture.

(1) We have used dual-parameter flow microflorimetry to quantitate the appearance of neoplastic 2-10-1 RTE cell populations from clonal progeny of early passage (preneoplastic) cultures. These studies show that many, if not all, cells in preneoplastic cultures possess the ability (are programmed) to convert to a particular neoplastic phenotype, and that they do so after a relatively constant number of population doublings.

(2) We have studied the growth interaction of neoplastic passage cells mixed with preneoplastic cells (early passage). Neoplastic cells rapidly become the dominant cell type. In addition to slight growth advantages of doubling time and plating efficiency that neoplastic cells enjoy, they in some way inhibit growth of the preneoplastic cells, possibly through direct cell-cell interaction.

(3) The development of heteroploidy and the expression of rat tracheal epithelial antigens were studied in spontaneously and chemically transformed RTE cell cultures. Most of the immortalized RTE cell cultures had elevated DNA contents with model DNA values ranging from 3.0-6.4C, compared to values of 2.1-2.3C for normal primary RTE cell cultures. In addition, all spontaneous and chemically induced RTE cell transformants exhibited increased antigen levels, when compared to levels detected on primary RTE cell cultures by indirect immunoassay using radioisotope or fluorescent methods. Neoplastic transformants appeared to exhibit higher antigen levels than preneoplastic variants. The evolution of cell types having heteroploid DNA content and increased cell surface antigen in short-term primary RTE cell culture was observed by simultaneous 2 parameter analyses of individual cells stained for both antigen and DNA content. The data indicate that spontaneous as well as induced RTE cell transformants undergo similar changes during the process of immortalization/transformation.

We have tested for binding of MoAb to RTE antigens on fibroblasts transformed with Rauscher leukemia virus. More than 10 cell lines were tested against a panel of eight MoAb. While significant binding was detected for some MoAb to certain cell lines, no correlation between binding of a particular MoAb and malignancy of the particular cell line was noted.

PUBLICATIONS

Braslawsky, G.R., Kennel, S.J., and Nettesheim, P.: Phenotypic changes in epithelial cell populations undergoing neoplastic transformation in vitro. In Cohn, W.E. (Ed.), Progress in Nucleic Acid Research and Molecular Biology, Vol. 29. Academic Press, Inc., New York, 1983, pp. 262-265.

Kennel, S.J., Foote, L.J., Lankford, P.K., Johnson, Marynelle, Mitchell, Tohy, and Braslawsky, G.R.: Direct binding of radioiodinted monoclonal antibody to tumor cells: Significance of antibody purity and affinity for drug targeting or tumor imaging. Hybridoma 2: 297-310, 1983.

Nettesheim, P., Braslawsky, G.R., Steele, V.E., and Kennel, S.J.: Cell population studies during epithelial carcinogenesis. In Owens, A.L., Jr., Coffey, D.S., and Baylin, S.B. (Eds.), Tumor Cell Heterogeneity: Origins and Implications, Vol. 4. Academic Press, New York, 1982, pp. 301-317.

LABORATORY OF REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY

LABORATORY OF REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY
Summary Statement

Because the detection of environmental agents which affect reproduction or produce birth defects is unsure and the underlying biological mechanisms which account for these major health problems are unclear, the Laboratory of Reproductive and Developmental Toxicology seeks to bridge the gap between the most molecular aspects of reproductive and developmental biology and endocrinology and the more applied problems associated with the detection of hazardous chemicals, the extrapolation of laboratory data to humans, and the estimation of human risk.

The Laboratory directs its research efforts to three major areas at the present time: (1) male and female reproductive processes; (2) developmental biology and endocrinology; and (3) toxicology/pharmacology. It is organized into four research sections: Developmental Endocrinology and Pharmacology Section (DES), Experimental Teratogenesis Section (ET), Gamete Biology Section (GB), and Reproductive Neuroendocrinology Section (RN). Extensive collaboration exists between these groups; thus, the current research projects for the Laboratory are listed below as integrated programs at various levels.

I. MOLECULAR LEVEL

A. Hormone-Related Gene Action in Accessory Sex Organs

- o Hormone regulation of protein synthesis in the prostate, seminal vesicle and uterus
- o Androgen-dependent sperm surface modification in the epididymis
- o Molecular characterization of androgen dependent rat and mouse seminal vesicle genes

B. Hormone Regulation of Embryonic and Fetal Gene Expression

- o Normal and glucocorticoid influenced expression of secretory protein genes in developing liver and yolk sac
- o Molecular characterization of glucocorticoid responsive genes
- o Identification of putative regulatory DNA regions via DNA-mediated gene transfer and *in vitro* hormone-receptor binding
- o Glucocorticoids and oncogene expression

C. Biochemical Basis of Estrogen Action

- o Characterization of nuclear events involved in estrogen action in the mouse uterus
- o Structural requirements for estrogen activity with emphasis on diethylstilbestrol and its metabolites
- o Analysis of proteins of the mouse uterus which are involved in estrogenic responses
- o Differential uterine cell responsiveness to estrogen
- o Cellular mechanisms of acute estrogen negative feedback on LH secretion

D. Biochemical Basis for Normal and Abnormal Craniofacial Development

- o Glucocorticoid involvement in prenatal craniofacial development
- o Role of adult and embryonic epidermal growth factor (EGF) in prenatal growth and differentiation
- o Biochemical basis of TCDD teratogenesis
- o Role of inositol-containing lipids

E. Biochemical Basis of Peptide Hormone Action

- o Characterization and analysis of peptide hormone receptors in anterior pituitary cells
- o Biochemical mechanisms regulating peptide hormone release at the synaptic level
- o Peptide-amine interactions modulating pituitary hormone release

F. Neurochemical Basis of Sexual Differentiation

- o Organizing effects of steroid hormones on hypothalamic neuronal systems regulating gonadotropin secretion

G. Neurochemical Basis of Developmental Changes in Neuroendocrine Regulatory Systems

- o Neurochemical correlates and role in the maturation of the neuroendocrine stress response during development
- o Evaluation of the developmental and neurochemical events underlying the neurotoxin-induced hypothalamic hypopituitarism
- o Neurochemical regulation of the neuroendocrine responses occurring during lactation/suckling, pregnancy, and the normal estrous cycle of the rat

H. Synthesis of Spermatogenic Cell Membrane Antigens

- o Protein synthesis by isolated spermatogenic cells
- o Stage-specific expression of cell surface carbohydrate antigens during spermatogenesis
- o Immunochemical characterization of stage-specific surface antigens during spermatogenesis

I. Identification of Developmental Lineages

- o Cell surface markers of pluripotent cells

II. ULTRASTRUCTURAL/CELLULAR STUDIES

A. Ultrastructural Changes as Predictors of Functional Abnormalities

- o Correlation of scanning and transmission electron microscopy observations with biochemical, histological, or functional changes in the male and female mouse genital tract as well as cells in culture

B. Physiology and Pharmacology of Amine Metabolism by Target Tissues

- o Uptake and metabolism of biogenic amines by neuroendocrine tissues. Role of steroid hormones
- o Source of monoamines involved in the regulation of pituitary hormone release

C. Toxication/Detoxication of Environmental Chemicals by Target Tissues Related to Reproduction

- o Potentiation of TCDD-induced transcription of prostatic cytochrome P1-450 mRNA following treatment with DNA modifiers
- o Characterization of diethylstilbestrol and steroidal estrogen metabolism and the elucidation of metabolic pathways which produce metabolites of differing biological activities
- o Elucidation of the interaction of estrogen metabolism and prostaglandin biosynthesis

III. CELL/TISSUE/EMBRYO STUDIES

A. Sperm

- o Protein analysis of epithelial cells from precaput, caput, corpus and cauda epididymides to identify sperm maturation factors
- o Rat epididymal sperm monoclonal antibodies
- o Epididymal secretion of a mouse sperm surface component

- o Loss of a sperm surface antigen coincident with capacitation
- o Participation of sperm surface molecules in fertilization

B. Cultured Embryos

- o Establishment and biochemical/physiological/morphological characterization of an in vitro system to grow and maintain whole rodent fetuses during critical periods of organogenesis

C. Isolated Development of Fetal Organs

- o Establishment and biochemical/physiological/morphological characterization of an in vitro system to grow and maintain fetal mouse genital tracts and gonads during the period of estrogen sensitivity
- o Morphological and functional characterization of heterologous cultures of testes and Mullerian ducts derived from DES-exposed and unexposed fetal mice
- o Morphological characterization of long-term fetal tissue grafts

D. Cell Cultures as Model Systems

- o In vitro neoplastic transformation of embryonic cells by diethylstilbestrol (DES) and structurally or functionally related chemicals
- o In vitro growth and differentiation of palatal epithelial cells
- o Hormonal control of differentiation and proliferation of seminal vesicle epithelial cells in collagen gel cultures
- o Hormonal control of differentiation and proliferation of uterine epithelial cells in collagen gel cultures
- o Gene expression during embryonal carcinoma cell differentiation in vitro as a model for early embryonic development
- o Prepuberal spermatogenic cells in short-term cultures
- o Short-term cultures of median eminence tissues and characterization of acute in vitro tests to elucidate direct hypothalamic effects of different agents
- o Autoradiographic analysis of peptide receptors in pituitary cells
- o In vitro culture and superfusion of enriched cell fractions of dispersed pituitary cells

IV. WHOLE ANIMAL STUDIES

A. Physiology

- o Role of neural peptides on testicular function
- o Central and gonadal effects of LHRH analogs
- o Integrated neuroendocrine stress response: role of central monoaminergic and peptidergic systems

B. Toxicology

- o *In vivo* studies on the teratogenicity of TCDD and glucocorticoids
- o Characterization of reproductive tract function (including fertility and carcinogenicity) in male and female mice exposed *in utero* to diethylstilbestrol

C. Data Extrapolation to Man and Risk Estimation

- o Diethylstilbestrol-exposed mouse offspring as a model for similarly exposed humans
- o Short-term *in vitro* assay for teratogens using human embryonic cells
- o The use of the monosodium glutamate-treated rat as an animal model for human hypothalamic hypogonadism

Summaries of these projects are presented below: details of the work appear in the individual annual reports.

I. MOLECULAR STUDIES

A. Hormone-Related Gene Action in Accessory Sex Organs

Hormone regulation of protein synthesis in the prostate, seminal vesicle and uterus: Protein synthesis patterns analyzed by two dimensional (2D) gel electrophoresis in the prostate and seminal vesicle of castrated and testosterone stimulated rats indicate that a major group of secretory proteins in both organs is under androgen control. Both organs have a high concentration of poly(A^+)-mRNA which code in a wheat germ translation system for major polypeptides. Two major poly(A^+)-RNA's from prostate (labeled β and δ) code for the subunits of the major secretory product referred to as prostate binding protein or prostatein. These two prostate poly(A^+)-mRNA make up 30-40% of the total poly(A^+) of the prostate. A third major prostate poly(A^+)-mRNA (α) codes for a larger (22,000 dalton) secretory protein. Likewise, rat seminal vesicles have two major poly(A^+)-mRNA's (40%) which code for two major seminal vesicle proteins which are androgen responsive (IV and V). (DES)

Androgen-dependent sperm surface modification in the epididymis: Epididymal maturation of sperm is androgen-dependent and sperm maturation antigen (SMA) 6 is significantly affected by castration, in that the percentage of sperm binding a monoclonal antibody to SMA 6 was reduced compared to sperm from control mice with ligated ductuli efferentes. Androgen treatment prevented the castration-induced changes of SMA 6, dihydrotestosterone being more effective than testosterone. In normal mice, SMA 6 progressively appears on sperm as they pass through the corpera and caudae epididymides and is present on sperm from mice and rats but is not present on sperm from rabbits and humans. SMA 6 is resistant to trypsin, but it is sensitive to neuraminidase and may be extracted from sperm with nonionic detergent, butanol, or chloroform:methanol (2:1). Solvent partitioning behavior suggests that SMA 6 may be a ganglioside. (GB)

Molecular characterization of androgen dependent rat and mouse seminal vesicle genes: We have previously reported the DNA sequence of a 3.3 kb Eco R1 fragment which contains the entire transcription unit plus 5'- and 3'- flanking regions of the rat seminal vesicle IV gene (SVS IV). The 5'- flanking region of this gene contains a perfect inverted repeat at -117 bp from the CAP site. The potential for forming a true cruciform structure in this region can be demonstrated in supercoiled configuration. Therefore, by S_1 nuclease-digestion of the supercoiled plasmid pSVS 3.3 (containing SVS IV), we have shown that there is a S_1 nuclease-sensitive site near the putative cruciform structure. We also detected an S_1 nuclease-sensitive site in the 3' flanking region of SVS IV about 800 bp from the termination site of the transcription unit. Recently we studied the S_1 nuclease-sensitive and DNAase I hypersensitive sites in the chromatin of the SVS IV gene. Nuclei from rat seminal vesicle were digested with S_1 nuclease or DNAase I. The DNA was then extracted and further digested with restriction endonucleases. DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters. Blots were hybridized with an SVS IV gene probe. The results indicated that the region around -100 to -150 bp was susceptible to the S_1 nuclease. DNAase I hypersensitive sites of SVS IV genes were present in seminal vesicle cells in which the gene is expressed and were absent in liver cells in which the gene is repressed. SVS IV gene is undermethylated in seminal vesicle and is highly methylated in liver. The present finding correlated well with the current hypothesis that the presence of DNAase I hypersensitive sites is necessary for transcription by RNA polymerase II *in vivo* and the actively expressed gene is generally undermethylated. (DES)

B. Hormone Regulation of Embryonic and Fetal Gene Expression

Normal and glucocorticoid influenced expression of secretory protein genes in developing liver and yolk sac: The endoderm cells of the visceral yolk sac are responsible for the synthesis of several proteins found in fetal serum and amniotic fluid, notably alphafetoprotein (69,000 dalton), metallothionein (6,000 dalton) and transferrin (75,000 dalton). Synthesis of these proteins is confined to visceral endoderm cells prior to liver differentiation, when fetal hepatocytes become the second cell type to produce these proteins. Transcription of the alphafetoprotein and metallothionein genes can be regulated by glucocorticoid hormones in neonatal and adult liver. However, it is not known if glucocorticoids can influence expression of these genes in the embryo or fetus. Glucocorticoid effects on transcription of these genes are

being studied by assaying for alphafetoprotein and metallothionein mRNA using cloned cDNA probes. Synthesis of these proteins is being studied by gel electrophoresis of labelled proteins extracted from short-term organ culture or from a reticulocyte lysate in vitro translation system. (ET)

Molecular characterization of glucocorticoid responsive genes: Genes being actively transcribed are thought to differ structurally from inactive genes. Active genes being more sensitive to nuclease cleavage and containing lower levels of the modified base 5-methylcytosine, the relationship between activity of the alphafetoprotein and metallothionein genes and their chromatin structure and base modification are being studied. The natural or chromosomal gene for alphafetoprotein has been purified and characterized by restriction endonuclease mapping and heteroduplex mapping. Various regions of this gene have been subcloned into plasmids and purified for use as hybridization probes and for sequence analysis. The metallothionein gene will be isolated from a λ charon 4A mouse gene library. (ET)

Identification of putative regulatory DNA regions via DNA-mediated gene transfer and in vitro hormone-receptor binding: The 5'-flanking DNA sequences of hormone responsive genes are involved in binding of hormone-receptor complexes and RNA polymerase II. These binding events may regulate qualitatively and quantitatively the transcriptional activity of hormone responsive genes. The 5 -flanking DNA of the AFP gene is being used to study binding of glucocorticoid-receptor complexes in vitro. Sequence analysis of this region coupled with deletion mutagenesis will allow for mapping of putative control regions for the AFP gene. Cultured cells can be transfected with foreign DNA. This DNA can be integrated into chromosomes and expressed by the recipient cell. The 5 -flanking DNA sequences of the AFP gene is being fused with the HSV-thymidine kinase gene and the fusion product introduced into a thymidine kinase deficient mouse cell line which contains glucocorticoid receptors. This system is being used to examine the involvement of specific sequences of the alphafetoprotein gene in response to glucocorticoid in vivo. (ET)

Glucocorticoids and oncogene expression: The expression of the cellular homologue (protooncogene) of various oncogenes has been linked to progression through the cell cycle. In addition, several polypeptide growth factors appear to stimulate tyrosine phosphorylation upon binding to their cell membrane receptors. These findings, taken together with the fact that a large number of the oncogenes code for tyrosine kinases, suggest that one way glucocorticoids may inhibit cell proliferation is via alteration of protooncogene tyrosine kinase expression. Recently, tyrosine kinases have been found to stimulate the phosphorylation of inositol-containing lipids (i.e., PI>PIP>PIP_2) which are thought to play a key role in regulation of cell proliferation. Since glucocorticoids alter both cell proliferation and PI metabolism, we are investigating effects of glucocorticoids on the expression of various oncogenes. (ET)

C. Biochemical Basis of Estrogen Action

Characterization of nuclear events involved in estrogen action in the mouse uterus: The second translocation of hormone receptor complex to the nucleus after exposure to estrogen, which occurs in the mouse uterus, suggests two events in estrogen receptor action. Compounds with poor estrogenic potency lack the ability to elicit this second nuclear event. The role of this event

in estrogen action in the mouse reproductive tract, with particular regard to the actions of hormonally active environmental chemicals, is being studied. Receptor synthesis, RNA polymerase activities, DNA polymerase activities and glucose oxidation/utilization are also being investigated. Progesterone inhibition of uterine growth is also being studied to determine the biochemical events involved in estrogen growth promotion. Recent studies using steroid autoradiography raise the possibility that the second nuclear accumulation of estradiol-receptor complex may be related to its redistribution to different cell types within the tissue. Thus, estrogen action in the mouse uterus may involve sequential stimulation of various tissue compartments for its expression. (DES)

Structural requirements for estrogen activity with emphasis on diethylstilbestrol and its metabolites: In order to determine whether the metabolism of DES resulted in biologically active or inactive metabolites, certain DES metabolites and analogs were tested for estrogenic activity using both an in vivo uterine bioassay and an in vitro receptor binding assay. Results of these studies showed good correlation between the biochemical and bioassay data. Compounds such as DES-epoxide or catechol-DES were associated with reasonable receptor binding and biological activity while certain metabolites, e.g., β-dienestrol or 1-hydroxy dienestrol, showed weak receptor interactions and poor estrogenicity. This indicates that the metabolism of DES does not result in complete inactivation. The exception to these results were some indenestrol isomers and ψ-DES isomers, which are possible DES metabolites and which show receptor binding comparable to DES, but were 20-100 times less biologically active. Studies are continuing to determine if the DES compounds have any influence on neuroendocrine activity. Preliminary results indicate that the indenestrol isomers show differences in their ability to suppress LH secretion. Testing of other compounds will be performed to determine if the same degree of biological activity exists at this in vivo estrogenic target site. The ψ-DES exists in E and Z isomeric forms having subtle structural differences; the hormonal activity of the separate isomers was tested to determine which form may be active. Receptor binding, nuclear translocation and tissue response data (growth, DNA synthesis, etc.) indicate both isomers have less activity than DES. The E-isomer has significantly lower activity than the Z-isomer. This difference may be explained by the fact that although the isomers bind similarly, the E-isomer does not translocate receptor to the nucleus as effectively. Studies are continuing with those compounds by investigating their X-ray crystal structure compared to DES to determine if structural and conformational forms of the compounds differ significantly. (DES)

Analysis of proteins of the mouse uterus which are involved in estrogenic responses: A molecular marker for estrogenic activity in uterine tissue is being sought to determine the activity and mechanism of action of hormonally active chemicals. Protein labeling experiments using [^{35}S] methionine have illustrated several proteins (32,000 - 54,000 mw range) in uterine tissue from estrogenized animals. Non-enzymatic separation of the three uterine tissue compartments have indicated that some of these proteins are unique to one cell type. These gels will be computer analyzed for qualitative and quantitative differences to determine estrogen responsive protein domains which may exist in different uterine cell types. Recently, we have purified a estrogen responsive secretory protein from mouse uterus through HPLC. This protein is highly glycosylated. Two-dimensional gel electrophoresis indicated this estrogen stimulated protein has molecular weight of 70,000 with pI 6.5-6.8.

Antibody against 70K protein has been raised in rabbits and a radioimmunoassay for this protein has been established. Studies are in progress to identify and determine a functional role for these proteins as well as to identify the tissue specificity and their possible physiologic roles. Polyclonal antibodies to this protein will be used to probe the ontogeny of this hormone response as well as study the hormonal regulation of this secretory product. (DES)

Differential uterine cell responsiveness to estrogen: In order to understand the tissue responsiveness to estrogens in more detail, we investigated the mitogenic action in different uterine cell types. Studies indicated that estradiol induces DNA synthesis and mitosis in uterine stroma and epithelium of sexual immature animals; while, mature adult animals show the mitogenic response only in the epithelium. Loss of stromal mitogenesis occurs between day 28-35 of development and coincides with sexual maturity. Experiments using intact and ovariectomized animals indicate this change can be influenced by the ovary. Studies will continue in order to determine and identify what ovarian substances may be involved in this effect. Epidermal growth factor (EGF) receptors are being assessed in uterine tissue to determine if EGF may play a role in the mechanism of estrogen induced uterine mitogenesis. Results indicate specific EGF receptor binding is present in uterine tissue. Further studies to determine the exact cellular localization of these EGF receptors and whether estrogens influence their expression or activity are underway. (DES)

Cellular mechanisms of acute estrogen negative feedback on LH secretion: In order to examine the cellular mechanisms by which estradiol (E_2) exerts its acute negative feedback effects upon luteinizing hormone (LH) secretion, the following will be temporally correlated: (1) serum LH; (2) pituitary responsiveness to luteinizing hormone-releasing hormone (LHRH); (3) the translocation of E_2 receptors from cytosolic (CER) to nuclear (NER) receptors of the pituitary, preoptic hypothalamic area and the caudal hypothalamic area; and (4) norepinephrine (NE), Dopamine (DA) and 5-hydroxytryptamine (5-HT) metabolism in several discrete areas of the preoptic-hypothalamic region in either female animals on diestrous day 1 or females which are ovariectomized (OVX) on diestrous day 1 and 10 days later are given a s.c. injection of corn oil or of estradiol benzoate (20 µg in corn oil/rat). Preliminary results demonstrate that serum LH increases 10-12 fold 10 days following OVX compared to diestrous controls. The injection of E_2 did not affect LH concentrations at 30 minutes but decreased serum LH at both 60 and 180 min following its administration. Pituitary responsiveness to LHRH (measured as the increase in LH 10 min after i.v. injection of 100 ng LHR H/rat) was not altered at 60 min but was significantly decreased 180 min following E_2 injection. Thus, serum LH decreased prior to a detectable alteration in pituitary responsiveness to LHRH, suggesting that the decrease observed at 60 minutes in LH concentrations was due to a central mechanism. Studies are now underway to determine the effects of the E_2 injection on the translocation of E_2 receptors from the CER to the NER and the metabolism of NE, DA and 5-HT in discrete regions of the preoptic-hypothalamic area to examine possible neurochemical correlates for this centrally-exerted decrease in serum LH 60 minutes following the s.c. administration of E_2. Preliminary results suggest that the central (brain) and peripheral (pituitary) negative feedback effects of E_2 involve different mechanisms and can be temporally separated. (RN)

D. Biochemical Basis for Normal and Abnormal Craniofacial Development

Glucocorticoid involvement in prenatal craniofacial development: Glucocorticoids, such as dexamethasone and triamcinolone acetonide, are potent inducers of cleft palate in a variety of experimental animals. There are a number of mouse strains which are highly sensitive to glucocorticoid-induced cleft palate, i.e., A/J, and others that are relatively less sensitive, i.e., C57BL/6J. The biochemical basis for this strain susceptibility appears to be due, in large part, to elevated levels of glucocorticoid receptors in the developing secondary palate. The level of receptors in the day 13 mouse embryos was found to be highest in the secondary palate of all the tissues examined suggesting that glucocorticoids and their receptors play an important role in palatal development. Immunocytochemical studies have localized these receptors present in the palate mainly to the mesenchymal cells. Studies are in progress, using primary and established lines of human and mouse palatal mesenchymal cells along with analysis of labeled proteins using one-and two-dimensional PAGE, to ascertain glucocorticoid-increased or suppressed protein synthesis. (ET)

Role of adult and embryonic epidermal growth factor (EGF) in prenatal growth and differentiation: EGF is a potent polypeptide capable of stimulating proliferation and differentiation in a number of cell types in vitro and in vivo. Our studies have provided evidence for an embryonic form of EGF that appears at midgestation and is presumably important for the development of a number of tissues including the secondary palate. EGF does not appear to cross intact in the rodent visceral yolk sac or chorioallantoic placenta; and, therefore, the appearance of this embryonic EGF on day 11 to 12 of gestation in the mouse is presumably due to synthesis by the embryo. Immunohistochemical studies are in progress to ascertain the exact site(s) of EGF synthesis in the embryo. EGF appears to be essential in vitro for the growth and differentiation of the palatal epithelial cells especially the oral and medial epithelial cells. Studies are in progress to further define the role that EGF plays in palatal development using cell, organ and whole embryo culture. (ET)

Biochemical basis of TCDD teratogenesis: The dioxin, TCDD, is the most potent teratogen known for experimental animals; it induces relatively few types of congenital anomalies in the mouse including cleft palate. In addition to this tissue specificity, strain differences in response to TCDD-induced cleft palate in the mouse correlate well with elevated levels of TCDD cytoplasmic receptors in palatal shelves from sensitive strains of mice. Whole body autoradiographic studies demonstrate that labeled TCDD appears in the developing embryo, especially in the secondary palatal shelves. Immunohistochemical studies are planned to localize the TCDD receptors in the palate. Morphological analysis has shown that TCDD does not affect the growth of the palate in vivo, but instead appears to interfere with some aspects of medial epithelial cell adhesion and/or programmed cell death. Studies are in progress using SEM, TEM, and autoradiography to further define this effect on the palatal epithelium. Studies are also in progress using palatal epithelial cell culture to further analyze the alterations induced by TCDD. (ET)

Role of inositol-containing lipids: Recent evidence has linked the receptor-mediated degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) and/or phosphatidylinositol (PI) to the generation of intracellular secondary messengers thought to play a key role in regulation of cell proliferation.

Glucocorticoids, which induce cleft palate in rodents by inhibiting palatal mesenchymal cell proliferation, markedly alter normal PI degradation and synthesis in a cell line derived from human embryonic palatal mesenchyme. This alteration in PI metabolism correlates in both dose response and time course to glucocorticoid-induced inhibition of cell proliferation. More recently, we have shown this alteration in PI metabolism to be receptor mediated using inactive hormone analogues. Furthermore, we have shown the alteration in PI metabolism to be independent of extracellular Ca^{++} concentration, intracellular Ca^{++}-calmodulin complex formation, and intracellular cyclic AMP levels. The alteration in PI metabolism is exacerbated by lithium ion which is known to inhibit a key enzyme in the recycling of the inositol moiety into newly synthesized PI. This finding provides additional evidence that the hormone-induced alteration in PI metabolism is due to an increase in degradation of pre-existing PI. The correlation between glucocorticoid inhibited cell proliferation and alteration of PI metabolism has been strengthened by the finding that stimulators of proliferation which also stimulates the PI cycle (i.e., serum) are inhibited by glucocorticoids while stimulators of proliferation (EGF) which do not stimulate PI metabolism are not inhibited by glucocorticoids. Further investigation is directed toward determination of specific PI metabolizing enzymes that glucocorticoids may alter. In addition, the effect of glucocorticoids on PIP_2 levels are under current investigation. It is possible that glucocorticoids initially alter PIP_2 levels which result in a change in the equilibrium established between PI ⇄ PIP ⇄ PIP_2 and ultimately alter PI metabolism. The results of these investigations are expected to yield additional information on the mechanism by which glucocorticoids alter inositol-containing lipid metabolism. (ET)

E. Biochemical Basis of Peptide Hormone Action

Characterization and analysis of peptide hormone receptors in anterior pituitary cells: Specific membrane receptors mediate the effects of peptide hormones on anterior pituitary hormone release. Using either purified membrane preparations or intact cells prepared by dispersion with collagenase, the binding of different neural peptides such as angiotensin II, arginine, vasopressin, corticotropin releasing factor, luteinizing hormone-releasing hormone, etc., is analyzed and correlated with the biological activity of the hormone, under conditions which maximize hormone release, or in circumstances in which hormone release is diminished or impeded by the use of steroid or other hormones physiologically involved in the regulation of specific pituitary hormones. (RN)

Biochemical mechanisms regulating peptide hormone release at the synaptic level: Under controlled incubation conditions, the release of LHRH and other peptides from synaptic terminals in the median eminence of the hypothalamus is finely regulated by a variety of agents, including ions (CA^{+2}, K^{+}, Mg^{+} and Mn^{+2}) prostaglandins (PGE_2, monoamines (norepinephrine, dopamine, epinephrine) and nucleotides (cAMP, cGMP). The effect of PGE_2 has been well documented. The role of arachidonic acid and several of its major metabolites is being analyzed in detail. Similarly, the specific adrenergic receptors involved in the response of the LHRH neuron to NE and specific adrenergic agonists is now being evaluated. Possible direct effects of estradiol and other steroids at the synaptic membrane level are also presently being considered. (RN)

Peptide-amine interactions modulating pituitary hormone release: Recent studies have indicated that co-incubation of dispersed pituitary cells with monoamines (such as serotonin) and peptides (vasopressin) resulted in a significant potentiation of the effect of the peptide. This intriguing effect suggests that amines, in addition to having direct effects upon hormone release, can also interact in a selective fashion with specific neural peptides to modulate pituitary hormone release. The intimate mechanisms underlying this important type of interaction are being presently studied. (RN)

F. Neurochemical Basis of Sexual Differentiation

Organizing effects of steroid hormones on hypothalamic neuronal systems regulating gonadotropin secretion: Estrogens are important organizational agents within the central nervous system during the developmental stages of neuronal growth, neuronal migration, synaptogenesis, etc. Within the neuroendocrine system, estrogens are important determinants of sexual differences that, according to the species, appear at different intervals during the fetal and neonatal periods. We have determined that, in the rat, estrogens are responsible for an important sex difference, i.e., the response to removal of gonadal feedback, which shows a marked sexual dimorphism. Our studies have analyzed the neurochemical basis of this sexual dimorphism as well as the neurochemical substrate for the estrogenic effect. Focusing primarily on the hypothalamic arcuate nucleus, a distinct sex difference in the activation of the tuberoinfundibular dopaminergic system after gonadectomy can be observed. Further, the dichotomy in the response is present after sexual maturation has been completed. The effects of estrogens upon this system may involve the induction of progesterone receptors within specific neurons of the arcuate nucleus, and these receptors may, in turn, permit the establishment of the delayed pattern of response to removal of feedback inhibition characteristic of the female. (RN)

G. Neurochemical Basis of Developmental Changes in Neuroendocrine Regulatory Systems

Neurochemical correlates and role in the maturation of the neuroendocrine stress response during development: Acute stressful stimuli characteristically result in unambiguous increases in ACTH, β-Endorphin and prolactin(PRL). Parts of this typical response, which is fully expressed during adult life, are absent, or significantly attenuated, during the infantile period. We have correlated the lack of the PRL response during the infantile period with a parallel deficiency in the maturation of a stimulation of the serotonergic system in the medial basal hypothalamus which is functionally coupled to an endogenous opiatergic system. Possible cellular and subcellular mechanisms that might contribute to this phenomenon are the lack of fully developed synaptic contacts between serotonergic and opiatergic cells, a paucity of serotonin receptors in selected hypothalamic neuronal membranes, or a predominence of inhibitory opiatergic receptors. Recent evidence suggests that the opioid receptors already exist in connection with the serotonin neurons involved, that direct stimulation of the serotonin receptors can elicit the PRL response (so that a paucity of 5-HT receptors is unlikely), and that the action of stress or of morphine, itself, on PRL release both appear to act, at least in part, by disinhibiting the tonic inhibition by DA on PRL release from the anterior pituitary. Since the various hormonal responses

appear at different times during development, it appears that it may be possible to use this model to dissect the neuronal systems and the individual neurotransmitter system(s) involved in the release of particular pituitary hormones during stress. (RN)

Evaluation of the developmental and neurochemical events underlying the neurotoxin-induced hypothalamic hypopituitarism: Injection of a variety of neurotoxins during the neonatal period produces permanent damage in selected brain regions, which result in profound morphological, neurochemical, endocrinological, and behavioral alterations later during adult life. Using monosodium glutamate as a selective neurotoxin, we have characterized the neurochemical changes that mediate these pathological alterations. Profound damage to the dopaminergic, and to a lesser extent, noradrenergic and serotonergic systems within the hypothalamus, were observed. Not only concentrations of the amines in selected neuronal systems were altered, but also metabolism (evaluated by HPLC combined with electrochemical detection) as well as the subcellular readily-releasable pools of the amines, were affected. These alterations in monoamine metabolism with the consequent lack of proper stimulation of peptide hormone release may explain the marked increase in pituitary responsiveness to peptide hormones which we have described in this animal model. Additional studies are being performed to elucidate the intimate mechanism(s) which may mediate these phenomena. (RN)

Neurochemical regulation of the neuroendocrine responses occurring during lactation/suckling, pregnancy, and the normal estrous cycle of the rat: There exist dynamic changes in the secretion of gonadal hormones (prolactin, luteinizing hormone, and follicle-stimulating hormone) during the physiological states of pregnancy/pseudopregnancy; lactation, alone, or lactation with suckling stimulus, and throughout the different stages of the estrous cycle which are specific in terms of time of occurrence and duration, age of the animal and the types of hormones affected. Little is currently known about the neurochemical/neuropeptide components and interaction involved in these neuroendocrine responses and studies are currently being performed to evaluate the possible involvement, sites of action and/or interactions of norepinephrine, epinephrine, 5-hydroxytryptamine, dopamine, β-endorphin and LHRH neurons participating in these hormonal responses. (RN)

H. Synthesis of Spermatogenic Cell Membrane Antigens

Protein synthesis by isolated spermatogenic cells: Incorporation of ^{35}[S]methionine into TCA-precipitable counts was compared for spermatogenic cells isolated from prepuberal and adult mice and maintained in short term cultures. Cell suspensions from 17 day animals contain early meiotic germ cells (preleptotene to early pachytene spermatocytes) and Sertoli cells, while cell suspensions from adults contain late pachytene spermatocytes, spermatids and residual bodies. Cells from 17 day mice incorporate more than twice the amount of radioactivity on a dpm/cell basis as cells from adults throughout an 8-hour incubation period. Autoradiograms of two-dimensional gels demonstrate incorporation of ^{35}[S]methionine into > 300 proteins (M_r 20,000-150,000, pI 4.5 - 7.5). The majority of these proteins are detected on autoradiograms and silver stained gels of both cell populations, although quantitative and qualitative differences occur. Prior studies have demonstrated the appearance of cell surface antigens in a defined temporal sequence, using monoclonal and polyclonal antibodies against purified populations of spermatogenic cells or spermatozoa.

A number of these antigens are synthesized in a stage-specific manner as demonstrated on two-dimensional autoradiograms from five purified germ cell populations (preleptotene, leptotene/zygotene, 17 day pachytene and adult pachytene spermatocytes, and round spermatids). These results suggest that biochemical and immunological approaches can be used to determine the synthetic profiles for individual proteins throughout spermatogenesis as a means of monitoring stage-specific metabolic processes and their perturbations by experimental treatment. (GB)

Stage-specific expression of cell surface carbohydrate antigens during spermatogenesis: Carbohydrate antigens have been implicated in cell and tissue specific adhesion and recognition events. Monoclonal antibodies (C6, A5, and J1) have been prepared which recognize three cell surface carbohydrate antigens present on dissociated prepuberal and adult testicular cells of the mouse and are expressed in a stage-specific manner on early pachytene spermatocytes. The antigenic determinants have been partially characterized using a sugar hapten inhibition assay. The binding of the three monoclonal antibodies is inhibited by N-acetylglucosamine, N-acetyllactosamine and lactose, and N-acetyllactosamine, lactose, and D-galactose, respectively. The antigenic determinants are present on glycoconjugates of high apparent molecular weight as determined by enzyme immunoassay of fractions from gel filtration columns and immunostaining of Western blots of SDS-polyacrylamide gels. These results indicate that keratin-like cell surface molecules of high molecular weight are synthesized in a stage-specific manner during an early phase of meiosis. Neuraminidase treatment of sections of prepuberal and adult testes exposed antigens on cells in earlier stages of spermatogenesis, suggesting that changes in cell surface sialylation is one mechanism regulating the expression of these antigens. (GB)

Immunochemical characterization of stage-specific surface antigens during spermatogenesis: Spermatogenic cell antigens appearing on the cell surface during the pachytene stage of meiosis have been identified immunochemically using polyclonal antisera prepared against mouse germ cells at defined stages of spermatogenesis. Four antisera prepared previously against purified populations of spermatogenic cells from the adult testes and excurrent ducts were used to stain Western blots from one- and two-dimensional SDS polyacrylamide gels of germ cell plasma membranes. Three antibodies (AP, ARS, ASC) recognize a similar subset of high molecular weight antigens plus a number of minor constituents which vary qualitatively and quantitatively between antisera. AVDS, prepared against vas deferens sperm, exhibits the least complex binding pattern. These results indicate that at least ten surface constituents appear during the pachytene stage of meiosis, a period where maximal RNA and protein synthesis has been demonstrated. Furthermore, two-dimensional immunoblot comparisons of plasma membranes isolated from late pachytene spermatocytes and round spermatids reveal differences between surface determinants detectable at these two spermatogenic stages. Antisera prepared against spermatogenic cell populations isolated from prepuberal mice have also been utilized to examine transitions in surface constituents during the early stages of meiosis. Immunofluorescent binding studies indicate that ALZ, raised against a population of leptotene and zygotene spermatocytes, binds to the surface of early pachytene spermatocytes and germ cells at subsequent stages of differentiation. Stage-specific antigens recognized by ALZ, including both protein and probable lipid, have been localized immunochemically on Western blots from SDS gels. A dithiothreitol-sensitive constituent

(M_r ~39,000) has been identified as the major protein determinant present in early meiotic cells but absent in spermatogonia from 8-day-old animals. This antigen is present in preleptotene, leptotene/zygotene, and early pachytene spermatocyte populations. This constituent could remain within the cell during the early meiotic stages and then be incorporated into the plasma membrane during pachynema, thus accounting for the stage-specific surface labeling observed with ALZ. These studies have identified a number of surface constituents that are not shared by somatic cells and are expressed during restricted periods of spermatogenesis. Such stage-specific macromolecules are candidates for further study using monoclonal antibodies to address functional questions concerning cell-cell interactions during germ cell differentiation. (GB)

I. Identification of Developmental Lineages

Cell surface markers of pluripotent cells: Pluripotent cells capable of forming all fetal tissues have been identified in mammalian embryos from the beginning of cleavage until the time of primitive streak formation, and primordial germ cells which give rise to the germ line are recognizable shortly thereafter. Two monoclonal antibodies (EMA 1 and 6) prepared against teratocarcinoma cells react with cell surface antigens on early mouse embryos and primordial germ cells. These antigens first appear at the 8-cell stage of development and persist through blastulation, becoming restricted in distribution to cells of the embryonic ectoderm of primitive streak stage embryos, before the appearance of primordial germ cells. The antigens are useful markers for identifying and monitoring pluripotent embryonic cells, primordial germ cells, and teratocarcinoma cells, but are not gene products specific to totipotent cells. The monoclonal antibodies also react with sperm and cells of the decidual mass, endometrium, brain, kidney, and epididymis. Although prior studies have assumed that antigenic determinants recognized by monoclonal antibodies in such cases are carried by cell surface molecules common to the various cell types involved, they may also represent structurally identical domains on different cell surface molecules. This system is being used to examine the nature of the molecules on different cell types to determine which of these interpretations is correct. (GB)

II. ULTRASTRUCTURAL/CELLULAR STUDIES

A. Ultrastructural Changes as Predictors of Functional Abnormalities

Correlation of scanning and transmission electron microscopy observations with biochemical, histological, or functional changes in the male and female mouse genital tract as well as cells in culture: Studies have demonstrated that scanning electron microscopy (SEM) provides a tool for the detection of early neoplastic changes. The surface ultrastructural features of the lumen of the female mouse genital tract was evaluated during the estrous cycle and during development in normal CD-1 mice. The hormone dependence of fine structural features of the cell surface was demonstrated in ovariectomized, hormone-treated females where various characteristics of intact animals were experimentally induced. Subsequent studies on DES-treated mice indicate that cell surface features are directly related to abnormal cell differentiation.

Changes in the cell surface are correlated with alterations in the histological features of DES-exposed offspring. Transmission electron microscopic studies have shown that the most striking and reproducible ultrastructural lesion in the uteri of prenatally-DES treated females is an abnormal stromal-epithelia interface. Experiments are underway to characterize by SEM and TEM the fine structure of mouse genital tract cells grown in culture. (DES)

B. Physiology and Pharmacology of Amine Metabolism by Target Tissues

Uptake and metabolism of biogenic amines by neuroendocrine tissues. Role of steroid hormones: The way amines are metabolized by target tissues may determine to what extent the particular amine plays a modulatory role upon a given cell system. We are interested in determining the specific metabolic pathway(s) of serotonin, norepinephrine, epinephrine and dopamine in key neuroendocrine tissues, such as hypothalamus, median eminence, anterior and posterior pituitary. We employ very sensitive electrochemical techniques to measure concurrently the amines, their respective metabolites and amino acid precursors. We also measure the activity of specific enzymes involved at various levels of the monoaminergic biosynthetic pathways. In addition, the role of steroids, particularly estrogen, on the induction or modulation of some of those enzymes (such as L-AAD) is also being evaluated. (RN)

Source of monoamines involved in the regulation of pituitary hormone release: Evidence from a number of laboratories including our own has demonstrated the capability of several monoamines, especially dopamine, epinephrine, and 5-hydroxytryptamine to alter the secretion of a number of pituitary hormones, including prolactin, directly at the level of the anterior pituitary. Such evidence emphasizes the importance of identifying the location of the source of supply of these monoamines to the anterior pituitry. Possible sources include: (a) the hypophyseal portal vasculature connecting the medial basal hypothalamus and, in particular, the median eminence with the anterior pituitary; (b) tuberohypophyseal neurons innervating the neurointermediate lobe of the pituitary which may release the amines into the hypophyseal vasculature which could then carry them to the anterior pituitary; and (c) direct innervation of the anterior pituitary. Results to date demonstrate that, unlike the median eminence or neurointermediate lobe, the anterior pituitary lacks the capacity to decarboxylate the precursors of dopamine and serotonin, L-DOPA and L-5HTP, respectively, to the amine products and is, therefore, dependent on other sources to supply its quota of 5-HT and DA in the regulation of anterior pituitary secretion. These results prove that the anterior pituitary cells which have classically been categorized as part of the APUD system actually lack the capability to form the amines from their amino acid precursors. Further studies in our laboratory have demonstrated for the first time that epinephrine and 5-HIAA (the deaminated metabolite of 5-HT) are present in much higher concentrations in the hypophyseal portal plasma than in peripheral plasma, that the epinephrine in the portal plasma is primarily derived from a central source, and that the 5-HIAA is apparently released into the portal vasculature as 5-HIAA. These results are important in terms of recent evidence demonstrating an effect of epinephrine upon ACTH, prolactin and growth hormone directly at the anterior pituitary level. Furthermore, they suggest that a compound thought of as an end product metabolite of serotonin may have a functional role in terms of anterior pituitary function. Current studies are examining the possibility that 5-HIAA may alter anterior pituitary hormone secretion. Finally, the present data suggest that 5-HT,

unlike DA or epinephrine, may arrive at the AP via routes other than that of the portal circulation. Experiments utilizing lesions of the median eminence and transplantation of the anterior pituitary under the renal capsule or removal of the neurointermediate pituitary lobe in vivo may provide important evidence to determine whether a central source is important in supplying a large portion of the 5-HIAA found in the animal's endogenous anterior pituitary. (RN)

C. Toxication/Detoxication of Environmental Chemicals by Target Tissues Related to Reproduction

Potentiation of TCDD-induced transcription of prostatic cytochrome P_1-450 mRNA following treatment with DNA modifiers: Oral TCDD pretreatment results in a 200 fold increase in prostatic AHH activity. This induction is potentiated 5 fold by prior intraperitoneal treatment with a DNA damaging agent such as procarbazine. DNA probes (cDNA) were used to monitor the effects of inducer and inducer plus DNA damaging agents on transcription rate of cytochrome P1-450 mRNA. A careful study with respect to translocation of TCDD-cytosol receptor to prostatic nuclei demonstrated no statistical difference between the TCDD-alone and TCDD-plus DNA modifiers. Selective restriction enzyme digests of prostatic genomic DNA suggested potentiated transcription of P_1-450 mRNA appeared to be associated with demethylation of 5^{me}-cytidine of GCC^{me}-G sequence in P_1-450 structural gene. (GB)

Characterization of diethylstilbestrol and steroidal estrogen metabolism and the elucidation of metabolic pathways which produce metabolites of differing biological activities: It has been demonstrated that peroxidase, an enzyme inducible in estrogen target tissues, is able to metabolize DES to its major metabolite, β-dienestrol. Bioactivation of DES was determined by the non-extractable binding of radioactivity to DNA and protein after incubation of ^{14}C-DES with several activating systems including one derived from a target tissue, the mouse uterus. This peroxidase activating system was also studied in the hamster kidney, a non-genital target tissue for DES. The peroxidatic activity of prostaglandin synthetase was found to catalyze the oxidative metabolism of DES in vitro and in cell culture. Moreover metabolism of DES via the peroxidase pathway was demonstrated in isolated organ cultures of fetal genital tissues but not fetal livers. Current studies include similar metabolic approaches to steroidal estrogen and appear to follow pharmacologic principles established for DES. The estrogenic activities of a series of DES metabolites and analogs were determined. Results of these studies suggest that DES metabolism follows alternative pathways resulting in either metabolites which retain estrogenicity, lack activity or are of ambiguous activity. The latter class includes the indenestrol isomers and pseudo DES. These compounds have a comparable binding affinity to DES but are some 20-150 times less biologically active. Studies on the levels at which they fail to elicit a biological response include estrogen receptor translocation/clearance, DNA synthesis and mitosis. Determination of the biological significance of potentially activated metabolites of DES should aid in making generalizations to other classes of estrogenic environmental chemicals. (DES)

Elucidation of the interaction of estrogen metabolism and prostaglandin biosynthesis: Studies have demonstrated that during oxidative metabolism of estrogens by prostaglandin synthetase, concurrent increases in prostaglandin levels are seen. This provides a link for early effects of estrogens and induction of prostaglandins. (DES)

III. CELL/TISSUE/EMBRYO STUDIES

A. Sperm

Protein analysis of epithelial cells from precaput, caput, corpus, and cauda epididymides to identify sperm maturation factors: Two-dimensional protein patterns of epithelial cells from precaput and caput compared to cauda epididymidis were strikingly different. Because these differences in the protein patterns were reproducible, they might serve as markers for detecting alterations in epididymal cell function with respect to sperm maturation and for determining sperm specific surface proteins of epididymal origin. (GB)

Rat epididymal sperm monoclonal antibodies. EA6B, AD8B, and BH9C reacted at different sites of sperm from the precaput, caput, corpus, and cauda epididymidis. The EA6B monoclonal antibody reacted equally with the head, surface, and the entire tail in precaput sperm. However, as sperm traverse through the caput, corpus, and cauda epididymides, the tail demonstrated a greater fluorescence than the head. On the other hand, the AD8B monoclonal antibody reacted primarily with the entire tail and very weakly with the postacrosomal region and the BH9C monoclonal antibody exclusively reacted with the mid-piece region of sperm from corpus and cauda epididymides. The origins of the sperm surface antigens which specifically react with the three monoclonal antibodies will be determined by immunostaining of epididymal tissue sections and immunoblotting of 2-D gel from precaput, caput, corpus, and cauda epididymides. (GB)

Epididymal secretion of a mouse sperm surface component: New surface antigens recognized by monoclonal antibodies appear during sperm maturation in the epididymis. This might be due to insertion of components from the sperm cytoplasm, exposure or modification of pre-existing surface components, or attachment of new components to the sperm surface. Sperm maturation antigen (SMA) 4 appears on the flagellum of sperm as they pass from the caput to the cauda epididymidis. Sperm from the caput epididymidis do not undergo antibody-induced agglutination, while those from the cauda epididymidis agglutinate in the presence of antibody. Sperm retained in the ductuli effuentes following ligation do not acquire SMA 4, suggesting that its appearance is not due to changes intrinsic to the sperm and that the epididymal environment is required. Immunohistochemical studies on tissue from different regions of the male reproductive tract indicated that SMA 4 is a secretory product of the epididymal epithelium, produced in a short segment of the distal caput epididymidis. Ligation experiments showed that the absence of sperm or testicular fluid from the epididymis did not affect production of SMA 4. Examination of prepuberal mice indicated that antigen production is age dependent, production beginning in the epididymis of mice between two and four weeks of age. Immunohistochemical and immunofluorescent studies indicate that this antigen is not present in other tissues of the mouse and is not found on sperm from other laboratory animals or humans. By use of SDS polyacrylamide gel electrophoresis and immunostaining of Western blots, SMA 4 has been shown to have an apparent molecular weight of 54,000. The antigen is not detected in Coomassie blue or Amido black stained gels or blots, but is seen with PAS staining, suggesting a high degree of glycosylation. The 54,000

molecular weight antigen is present in sperm-free fluid from the cauda epididymidis but is not detected in extracts of sperm or in fluid from the caput epididymidis. These studies suggest that specific acceptor sites are present in the plasma membrane of the sperm flagellum which bind SMA 4 following its release from epididymal epithelium cells. (GB)

Loss of a sperm surface antigen coincident with capacitation: Sperm acquire the capacity to undergo the acrosome reaction while in the female reproductive tract or in in vitro fertilization medium and show a progressive loss of sperm maturation antigen (SMA) 4 coincident with time in these environments. SMA 4 is recognized by a monoclonal antibody and has been shown to be a molecule secreted by the mouse epididymis which binds specifically to the plasma membrane of the sperm tail during epididymal sperm maturation. Although SMA 4 is resistant to removal by washing and other treatments and appears to be bound to the plasma membrane by strong, non-covalent interactions, the antigen can be recovered from the incubation medium apparently intact following loss from the sperm surface. The time dependent loss of SMA 4 in vitro is inhibited by the presence of mouse or rat epididymal fluid, low incubation temperature, or lack of protein in the medium. Sperm which remain in the uteri of mice after natural or artificial insemination have SMA 4 on their surface, but sperm which traverse the oviduct and attach to cumulus cells or zonae pellucidae of eggs lack the antigen, suggesting that epididymal fluid concentration may affect loss of the antigen in vivo as well as in vitro. (GB)

Participation of sperm surface molecules in fertilization: During fertilization sperm are involved in multiple recognition and attachment processes, including interaction with cumulus cells, binding and penetration of the zona pellucida, and attachment and fusion with the egg plasma membrane. In vitro fertilization assays are being used to test the possible role of specific sperm surface molecules in these processes. Control studies have used mouse antiserum to mouse sperm to verify that syngenic, sperm-specific antibodies substantially inhibit fertilization. Hybridoma cell lines producing monoclonal antibodies recognizing mouse sperm surface antigens have been grown as ascites tumors and the antibody-containing ascites fluids used in the in vitro fertilization assays. Monoclonal antibodies binding to the same region of the mouse sperm surface have been compared in these assays and while some inhibit fertilization, others do not. This degree of specificity suggests that the in vitro fertilization assay is valuable not only for examining the putative role of particular sperm surface molecules in different cell recognition and attachment processes in reproduction, but may also be useful for testing the effects of genetic alterations of such molecules. (GB)

B. Cultured Embryos

Establishment and biochemical/physiological/morphological characterization of an in vitro system to grow and maintain whole rodent fetuses during critical periods of organogenesis: In vivo approaches to understanding the mechanisms of teratogenesis are useful, but limited, since manipulation of certain variables is not possible. In vitro culture of the whole mouse embryo is now possible in our lab during days 8-12 of organogenesis when the embryo is undergoing its most dramatic growth and organ development. Our results demonstrate that early somite (day 8) CD-1 embryos cultured in 100% rat serum for 48 hours with low oxygen tension grow and develop to the equivalent of day

10 embryos in vivo, whereas day 10 embryos cultured for 48 hours to the day 12 equivalent do best in high oxygen tension and 50% fetal calf serum. Studies are in progress to reduce the amount of serum necessary for whole embryo culture and develop a serum-free hormone-supplemented media for embryo culture. Glucocorticoids exert stage specific effects on embryonic development in culture: heart and neural tube malformations are induced between days 8 and 10 in culture; cleft lip is observed between days 10 and 12. Future studies will attempt to define the mechanism by which glucocorticoids are teratogenic in whole embryo culture. Studies are in progress to extend the time embryos are in culture in order to obtain complete development of the primary palate (day 10 to 12) and secondary palate (day 11 to 14). (ET)

C. Isolated Development of Fetal Organs

Establishment and biochemical/physiological/morphological characterization of an in vitro system to grow and maintain fetal mouse genital tracts and gonads during the period of estrogen sensitivity: The morphological and functional characterization of heterologous cultures of testes and Mullerian ducts derived from DES-exposed and unexposed fetal mice has been established. Explants of fetal mouse gonads and genital tracts maintained in organ culture have been used to determine protein maps of developing tissues derived from DES-exposed and unexposed animals. The biochemical studies are correlated with hormone response studies and morphological observations. Recent studies have focused on growth factor requirements for proliferative response to estrogens in vitro. These studies are augmented by 2d maps of the developing protein patterns associated with differentiation of these tissues. The oxidative metabolism of DES in the fetal genital tract organ culture system was determined; differences in metabolic patterns could be demonstrated in different fetal target tissues. (DES)

Morphological and functional characterization of heterologous cultures of testes and Mullerian ducts derived from DES-exposed and unexposed fetal mice: A heterologous organ culture system including DES-exposed (or unexposed) testes and/or Müllerian ducts has been used to determine the mechanism of Müllerian duct persistence and hyperplasia in DES treated males. Results suggest that the primary site of action is on the duct system rather than through a failure of the testis to synthesize or release Müllerian inhibiting hormone. (DES)

Morphological characterization of long-term fetal tissue grafts: To overcome difficulties in long-term (greater than two months) maintenance of fetal tissues in vitro, cultured fetal gonads or genital tracts are carried as grafts in appropriately manipulated hosts. Under these conditions, fetal ovaries develop into functional gonads when grafted under kidney capsules. Some of the epithelial abnormalities observed in vivo can be seen in long-term explants of fetal vaginal tissues. Thus, the contribution of the postnatal environment to expression of prenatally induced abnormalities can be studied. (DES)

D. Cell Cultures as Model Systems

In vitro neoplastic transformation of embryonic cells by diethylstilbestrol (DES) and structurally or functionally related chemicals: DES was shown to morphologically transform cells in culture at doses comparable to

benzo(a)pyrene. The cells were tumorigenic when injected into appropriate hosts. Transformation was accomplished in the absence of measurable somatic mutation and of stimulated cell proliferation. Chemicals with structures capable of bioactivation by the peroxidase pathway were efficient transformants. Metabolism of DES to β-dienestrol via this peroxidase pathway was shown to occur in this cell culture system. In the presence of exogenous metabolic activity systems, unscheduled DNA synthesis could be induced in these cell cultures by DES and some of its analogs. Perhaps most important is that DES (at transforming doses) was an efficient inducer of aneuploidy in these cells. Moreover, steroidal estrogens also induced transformation; catechol estrogens were more efficient than the parent compound. (DES)

In vitro growth and differentiation of palatal epithelial cells: Epithelial cell growth and differentiation in the secondary palate results in three distinct phenotypes: the ciliated mucous-secreting nasal cells; the squamous, keratinizing oral cells and the medial epithelial cells which are programmed to die. One of our major research goals is to understand what factors influence the normal and abnormal development of these phenotypes. We have developed in vitro conditions under which the growth and differentiation of the isolated epithelial cells can be examined. The day 12 to 13 palatal epithelium is enzymatically removed from the mesenchyme and allowed to attach to an extracellular-matrix coated culture dish rich in type I and III collagen and fibronectin. Growth factor and hormonal requirements have been determined by culturing in a serum-free medium. Epidermal growth factor is an absolute requirement for adhesion, growth and differentiation of the epithelial cells, especially the oral cells which seem to be the most responsive cell type. This is due, in part, to the fact that the oral cells have the highest amount of EGF receptors, as demonstrated using labeled EGF and autoradiography. This effect of EGF on the oral cells can be greatly enhanced either by adding cyclic AMP or co-culturing on lethally-irradiated 3T3 embryonic fibroblasts. It appears that there is a stem cell population in the oral epithelium, which under the appropriate conditions, can grow for long periods in culture. In the presence of EGF, the medial epithelial cells are affected by not undergoing programmed cell death while the nasal cells do not appear to respond to EGF. (ET)

Hormonal control of differentiation and proliferation of seminal vesicle epithelial cells in collagen gel cultures: Cell cultures in collagen gels with serum free media are used to determine the role of steroid hormones and peptide hormones in the differentiation (as determined by expression of the SVS IV gene and morphology) and proliferation of epithelial cells derived from the seminal vesicles of mice. Results demonstrate the requirement of EGF in proliferation of these epithelial cells. (DES)

Hormonal control of differentiation and proliferation of uterine epithelial cells in collagen gel cultures: Culture conditions for mouse uterine epithelial cells were determined using serum-free media and growth on collagen. As with seminal vesicle epithelial cells EGF supports maximal proliferation of these cells. Preliminary studies have shown that the conditioned media from mouse uterine epithelial culture contain the same proteins found as secretory products in the uterine luminal fluid of estrogen-stimulated mice. (DES)

Gene expression during embryonal carcinoma cell differentiation in vitro as a model for early embryonic development: An embryonal carcinoma cell line F9

derived from a mouse teratocarcinoma (germ cell tumor) can be induced to differentiate into two distinct populations of extraembryonic cell types, parietal and visceral endoderm. F9 monolayers treated with retinoic acid (Vitamin A) and dibutryl cyclic adenosine monophosphate differentiate into an early embryonic cell type parietal endoderm. F9 cells treated with retinoic acid in suspension culture, differentiate into aggregates called embryoid bodies. Embryoid bodies are so-called because they morphologically resemble early mouse embryos at the two-layered stage. Embryoid bodies synthesize large amounts of alphafetoprotein, transferrin and metallothionein which is characteristic of visceral yolk sac endoderm. This cell culture system is being exploited to analyze molecular mechanisms effecting expression of visceral endoderm specific genes. (ET)

Prepuberal spermatogenic cells in short-term cultures: To monitor synthesis and processing of stage-specific surface antigens during spermatogenesis, we have examined conditions for short-term culturing of germ cells isolated from adult and prepuberal mice. Pachytene spermatocytes and round spermatids from adults can be cultured with >90% viability for at least 24 h in Eagle's MEM supplemented with 10% fetal bovine serum (FBS), 6 mM lactate, 1 mM pyruvate. In contrast, cell suspensions from 17 day old (17d) testes, containing meiotic germ cells and Sertoli cells, decline to 60-70% viability when cultured under identical conditions. Viability of 17d germ cells did not improve in DMEM, F12, DMEM/F12, RPMI-1640, 199, or STIM-9. The use of FBS decreased viability of the 17d germ cells and was subsequently omitted. Improvements in viability were obtained by supplementing with growth factors and hormones. TK, an enriched MEM medium without serum developed for rat testicular cultures resulted in viabilities near 80% for 17d cells cultured for 16-20 h. Age differences were noted, with the viability of germ cells from 20d mice resembling those from adults, remaining in the 85-90% range for up to 40 h in MEM, CEM or TK. Like 17d germ cells, those from 18d or 19d mice had higher viabilities in TK than in MEM, suggesting that hormone and growth factor requirements may change markedly during this interval. The use of improved media such as TK for longer culture periods should facilitate studies of the synthesis of germ cell surface antigens and the regulation of their production in a stage-specific manner. In addition, these studies will facilitate the development of in vitro assays to monitor surface interactions between germ cells and Sertoli cells. (GB)

Short-term cultures of median eminence tissues and characterization of acute in vitro tests to elucidate direct hypothalamic effects of different agents: A standardized in vitro culture system employing specific hypothalamic fragments, such as the median eminence, can be used as a potential short-term test to evaluate the specific or nonspecific effects of drugs, chemicals or other potentially toxic agents upon the release of LHRH or other peptides and biogenic amines involved in the regulation of gonadotropin release. (RN)

Autoradiographic analysis of peptide receptors in pituitary cells: Autoradiographic studies are being performed with the aid of tritium-labeled peptides (such as ^{3}H-AVP) to evaluate receptor binding to specific anterior pituitary cells. Other factors such as receptor/peptide internalization are also being analzyed, as well as the receptor dynamics under conditions which stimulate hormone release in vitro. Further combined immunocytochemical staining will allow identification of the specific cellular type(s) showing autoradiographic evidence of binding. (RN)

In vitro culture and superfusion of enriched cell fractions of dispersed pituitary cells: Direct peptidergic and aminergic effects upon pituitary hormone release are mediated by intracellular messengers following binding to specific cell membrane receptors. Characterization and quantitation of these intracellular events is complicated by the presence of many different cell types in the pituitary. We will attempt to apply a method of cell enrichment separation based on an elutriation process, which has been successfully used to separate other cell types in peripheral tissues, including the testis. Upon collection of the enriched cell fractions, each population will be stimulated with appropriate secretagogues and the resulting hormone release correlated with intracellular changes in cyclic AMP/GMP, CA^{+2} mobilization, changes in arachidonic acid and phospholipid metabolism, etc. Furthermore, this system will be used to evaluate the possible role of 5-HIAA (deaminated metabolite of serotonin) in the regulation of anterior pituitary hormone secretion. (RN)

IV. WHOLE ANIMAL STUDIES

A. Physiology

Role of neural peptides on testicular function: Recent studies have characterized the presence in the testis of several species of a variety of neuropeptides such as arginine vasopressin (AVP), LHRH, β-Endorphin, ACTH, etc. We have confirmed and extended the observations on some of these peptidergic hormones (β-Endorphin, ACTH) and are currently evaluating the precise physiological role these peptides may play, what parameters do they regulate, and what factors modulate their release within the testis. (RN)

Central and gonadal effects of LHRH analogs: Recently developed superagonists of LHRH are now being successfully used as antifertility agents. Although some of the possible mechanisms of action have been described, it is still unknown, for instance, whether the analogs will act centrally in addition to their peripheral site(s) of action. Other possible effects of the analogs, particularly at the gonadal level, may not be restricted to direct inhibition of testicular synthesis but may also affect some paracrine actions of testicular peptides. A detailed evaluation of these central and gonadal effects of these analogs is being performed. (RN)

Integrated neuroendocrine stress response: role of central monoaminergic and peptidergic systems: Acute exposure to different stresses results in the release of several pituitary hormones, most notably prolactin, ACTH and β-Endorphin, and in decreases in the release of growth hormone and TSH. These changes are centrally-mediated and involve the release of the respective releasing and inhibiting factors which are in turn mediated by different monoaminergic systems. The multiple hormonal responses elicited by stress are integrated in many nuclei within, as well as outside, the hypothalamus. We are presently evaluating in detail the different components of this integrated neural response by employing refined microdissecting techniques as well as very sensitive assay systems for peptides and for monoamine content and metabolism. The results gathered provide information which can be used to build a map of the neurochemical events taking place in each neurotransmitter

system within individual hypothalamic nuclei during acute exposure to different stressful stimuli and aid in the discovery of the neural pathways involved in those stress-induced neuroendocrine events. (RN)

B. Toxicology

In vivo studies on the teratogenicity of TCDD and glucocorticoids: Glucocorticoids and TCDD are potent inducers of isolated cleft palate in various mouse strains. The mechanisms appear to be receptor dependent and involve a preferential inhibition of either craniofacial growth or interference with programmed cell death. Our studies have shown that vitamin B_6 in vivo can substantially reduce the incidence of glucocorticoid-induced cleft palate. It appears that excess vitamin B inhibits glucocorticoid binding to cytoplasmic receptors. Whole-body autoradiography, after administration of labelled glucocorticoids, demonstrates localization of glucocorticoid in the developing secondary palatal mesenchymal cells. There appears to be a synergistic interaction between TCDD and glucocorticoids with respect to cleft palate induction. When TCDD and glucocorticoids are co-administered on days 10-13 in the C57BL/6J mouse at doses which alone cause 7% and 18% cleft palate, respectively, the combination results in 100% cleft palate. Studies are in progress to examine various morphological and biochemical parameters in order to explain the basis for this synergistic teratogenic interaction. (ET)

Characterization of reproductive tract function (including fertility and carcinogenicity) in male and female mice exposed in utero to diethylstilbestrol: Prenatal exposure to DES in mice results in a dose-related decrease in fertility and genital tract abnormalities in the offspring of both sexes; in females, tumors of the vagina, cervix, uterus, and ovary were found. The stage of differentiation of the genital tract at the time of DES treatment was critical to the later expression of reproductive tract lesions. Induction of a benign vaginal lesion, adenosis, occurred later in development than the induction of the malignant vaginal lesion, adenocarcinoma. The oviducts of DES-treated mice were malformed (developmentally arrested) and developed progressive proliferative epithelial lesions, resulting ultimately in oviductal adenocarcinoma. Dysmorphogenesis of the female genital tract at the cellular level resulted in cellular defects in the ovary which contributed to altered gonadal steroid secretion. These studies should be pertinent to the development of an animal model for similar human exposures. (DES)

C. Data Extrapolation to Man and Risk Estimation

Diethylstilbestrol-exposed mouse offspring as a model for similarly exposed humans: Many of the genital tract lesions observed in mice exposed prenatally to DES have been observed in comparably exposed humans. For example, in the male, epididymal cysts, prostatic inflammation, sperm abnormalities, and cryptorchidism have been observed in both species; in females, vaginal adenocarcinoma has been seen in the prenatally-exposed mouse and human. Good examples of the utility of such studies is the report of retained testes in male mice derived from DES-treated mothers two years before a similar observation was reported in man and the report of dose-related subfertility and oviductal malformations in female mice two years before comparable reports in woman. Potency ratios during critical developmental periods in animals and humans are used to develop risk estimates and compare model fitness for selected DES-induced genital abnormalities. (DES)

Short-term in vitro assay for teratogens using human embryonic cells: Whole animal tests for teratogenesis are extremely expensive, time consuming, and inadequate to test all the chemicals now in existence and those introduced each year which may present a risk for the unborn. In vitro assays for teratogenesis would not eliminate animal testing but would serve to indicate which of the thousands of chemicals that have not been tested should have the highest priority for in vivo testing. We have developed a cell culture assay for teratogenesis. This assay is based on the sensitivity of a line of human embryonic palatal mesenchymal (HEPM) cells to chemically-induced growth inhibition over a four-day period in culture. Inhibition of growth of palatal mesenchymal cells by a number of diverse chemicals and drugs is the major mechanism by which cleft palate is induced in the rodent, and the currently available evidence suggests growth inhibition as a major etiology of human cleft palate. Therefore, these HEPM cells, established from a human embryonic palate at eight weeks of gestation, may be an ideal cell type to represent undifferentiated mesenchymal cells from a number of embryonic tissues which are frequently malformed in the human. To date, we have tested over 65 chemicals and drugs in which there is some information on the animal and/or human teratogenicity. There is a good correlation between the dose which causes a 50% inhibition of growth (ID_{50} and the known drug and chemical teratogenicity. Chemicals and drugs which are poor or weak teratogens have either no effect or an ID_{50} of 1mM or greater, whereas those which are proven teratogens in vivo have an ID_{50} of 1 nM to 100 μM. The capability of an in vitro system to metabolize proteratogens, such as cyclophosphamide, to teratogens (phosphoramide mustard) is a highly desirable feature. We have spent considerable time developing conditions for such an activation system. HEPM cells are directly exposed to arochlor-induced rat liver S-9 plus cofactors and teratogen for 4 hours during the day after the cells are seeded. Under these conditions, we obtain an S-9 dependent, dose-responsive inhibition of growth with cyclophosphamide (ID_{50} = 5 μg/ml). (ET)

The use of the monosodium glutamate-treated rat as an animal model for human hypothalamic hypogonadism: Neurochemical damage to selected brain nuclei induced by neonatal treatment with monosodium glutamate results in the development of many endocrine abnormalities leading to infertility in a large number of subjects. Many of the alterations resemble those seen in some human cases of hypothalamic hypogonadism. Further extensive testing of this animal model is being performed to evaluate fully the validity of using these animals as a model for studies evaluating the diagnostic and therapeutic approaches to the problem of infertility of hypothalamic origin. (RN)

COLLABORATIVE RESEARCH WITH ACADEMIC COMMUNITY AND GOVERNMENTAL AGENCIES

There are numerous examples of collaborative research projects at the local, national and international levels. Laboratory scientists appreciate the expertise and resources concentrated in nearby universities. Moreover, we have not hesitated to establish collaborative efforts with scientists throughout the world to increase the quality of intramural programs.

Dr. Andrews continues his efforts in a collaborative project with Dr. Eileen Adamston at the La Jolla Cancer Foundation in La Jolla, California, in which they are studying expression of genes during embryonal carcinoma cell differentiation. He is also collaborating with Dr. Lash Gedama at the University of Calgary, Calgary, Alberta, Canada, in a study of metalliothionein expression in embryonic mice.

Dr. Eddy is involved in collaborative research with Dr. Clifford Lingwood of the Sick Children's Hospital, University of Toronto, Canada, and Dr. Charles Muller of the University of Washington, School of Medicine, Seattle, on the metabolism and distribution of SGG, a glycolipid unique to the plasma membrane of spermatogenic cells.

Drs. Eddy and Toshimori are working in collaboration with Dr. Bruce Fenderson of the Fred Hutchinson Cancer Research Center, Seattle, on the fate of a sperm tail cytoskeletal element after fertilization.

Drs. Johnston and Negro-Vilar are involved in collaborative studies with Dr. Daniel M. Gibbs, Department of Reproductive Medicine, School of Medicine, University of California, San Diego, La Jolla, on the measurement of several monoamines and metabolites in the hypophyseal portal vasculature connecting the median eminence with the anterior pituitary.

Drs. Johnston and Negro-Vilar are also collaborating with Dr. John Reinhard, Department of Medicinal Biochemistry, Burroughs-Wellcome Company, on studies regarding the activity of the rate limiting enzyme for dopamine and norepinephrine synthesis, tyrosine hydroxylase, in the median eminence as well as the posterior and anterior lobes of the pituitary. In addition, a collaboration exists between Drs. Johnston and Negro-Vilar with Dr. Dana Vaughn, Department of Medicinal Biochemistry, Burroughs-Wellcome Company, on monoamine metabolism in discrete regions of the hypothalamus and the anterior pituitary as well as gonadotropin secretion in aging rats.

Drs. Johnston and Negro-Vilar collaborate with Drs. Richard H. Alper and Katherine Fagin, Department of Physiology, School of Medicine, University of California, San Francisco, on the pituitary content of various monoamines and metabolites in the median eminence and anterior pituitary as well as the secretion of ACTH, β-endorphin and prolactin into plasma following removal of the neuro-intermediate lobe of the pituitary under a variety of experimental manipulations in an attempt to decipher the source of those monoamines and metabolites found in the anterior pituitary.

Drs. Johnston and Negro-Vilar also collaborate with Dr. Klaus-Dieter Dohler at the Department of Clinical Endocrinology, Max-Planc Institute, West Germany, on the neurochemical correlates for the lack of a stress response for the hormone, prolactin, in a mutant strain of rats developed by Dr. Dohler.

Drs. Kim and Pratt are involved in collaborative studies with Dr. James Bawden, Dental Research Institute, University of North Carolina at Chapel Hill, concerning localization of labeled teratogens in the rodent embryo and with Dr. Jean Lauder of the Anatomy Department to localize glucocorticoid receptors using immunocytochemistry.

Dr. Korach remains involved in a collaborative research project with Drs. Indu Parikh and Bruno Moncharmont, Burroughs-Wellcome Research Labs, on estrogen receptor antibody immunoassay and antibody preparation; localization of estrogen receptor by antibody localization on two dimensional gels is also part of a collaboration with Dr. K. McCarty of Duke University.

Dr. Korach also continues to be involved in collaborative research studies with Dr. Jack Gorski at the University of Wisconsin (Madison) on the effect of DES

metabolites on rat uterine DNA synthesis and in collaborative research with Dr. Charles Eldridge at Bowman Gray School of Medicine regarding RIA of steroid hormone and luteinizing hormone levels. Dr. Korach continues his collaboration with Dr. Manfred Metzler on the DES metabolites and analogs.

Dr. Lee continues to collaborate with Drs. Daniel W. Nebert, Developmental Pharmacology, NICHD, and Carol Parker, LMB, NIEHS, to elucidate possible mechanisms of potentiation of TCDD-induced/P_1-450 gene by DNA methylating and demethylating agents.

Dr. McLachlan continues to be involved in collaborative research with Dr. Manfred Metzler of the Institute for Pharmacology and Toxicology, University of Würzburg, Germany; Dr. A. Haney, Director, Division of Reproductive Endocrinology, Department of Obstetrics/Gynecology, Duke University Medical Center; and Dr. B. C. Bullock, Department of Comparative Medicine, Bowman-Gray School of Medicine. These collaborative projects involve a detailed exploration of transplacental toxicity of DES and other hormonally-active chemicals.

Drs. McLachlan and Korach continue to participate in collaborative studies regarding X-ray crystallography of DES metabolites with Dr. William Duax, Medical Foundation of Buffalo.

Dr. Negro-Vilar is involved in a collaboration study with Drs. Fred Downey and M. T. B. deCastro from the Veterans Administration Hospital and Southwestern Medical School at Dallas, to analyze pulsatile secretion of ACTH and the neural and hormonal factors that control that pattern of release.

Dr. Negro-Vilar is also working in collaboration with Dr. E. Lapetina, Group Leader in the Molecular Biology Department at Burroughs Wellcome Research Laboratories, to evaluate transmembrane signaling and peptide hormone release. He collaborates with Dr. H. Viveros and Dr. David Duch from Burroughs Wellcome on studies analyzing the release of ACTH and presence of other peptides in a mouse tumor cell line (A^+T20) known to produce large levels of ACTH in culture.

Drs. Negro-Vilar and Valenca are involved in a collaborative study with Dr. C. L. Chen, The Population Council and Rockefeller University, to look at factors regulating the expression of proopiomelanocortin mRNA in the testis.

In addition, Dr. Negro-Vilar is performing studies in collaboration with Dr. M. Sar to evaluate peptide (vasopressin) receptor dynamics in anterior pituitary cells in culture in correlation with hormone (ACTH) release, using autoradiography.

Dr. O'Brien is studying the synthetic profiles of individual proteins during spermatogenesis in collaboration with Drs. Clarke Millette and George Gerton of the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston.

Dr. Pratt continues his collaborative studies on prenatal human development with Dr. T. Shepard, University of Washington; Dr. L. Dencker, University of Uppsala, Sweden, on the mechanism of TCDD-induced cleft palate; and Dr. V. Diewert of the University of British Columbia, Vancouver, on the mechanism of glucocorticoid-induced cleft palate.

Dr. Teng continues to be involved in collaborative research with Dr. Ching Sung Teng of the Department of Anatomy, Physiological Science and Radiology, School of Veterinary Medicine, North Carolina State University, on prenatal DES effect on specific gene activity and its future response to hormones. She also remains involved in a collaborative research with Dr. Stephen E. Harris, Recombinant DNA Group, W. Alton Jones Cell Science Center, Lake Placid, New York, on nuclease sensitivity of the SVS IV gene in chromatin of rat seminal vesicle after hormone stimulation and during development.

Drs. Valenca, Johnston, and Negro-Vilar are collaborating with Dr. Russel J. Reiter, Department of Anatomy, University of Texas Health Science Center at San Antonio, on the effects of neurotoxicity resulting from neonatal monosodium glutamate treatment on the content and circadian rhythym of melatonin in the pineal gland.

NATIONAL AND INTERNATIONAL PROGRAMS: SYMPOSIA ORGANIZED/COMMITTEE APPOINTMENTS, ETC.

Another important indicator of peer recognition and scientific relevance of current Laboratory programs is the frequency that LRDT scientists organize and participate in "state of the art" symposia and are asked to serve on various committees attempting to provide meaningful directions in environmental health research. Descriptions of representative examples for the Laboratory are given below:

Dr. Eddy was appointed a member of the Scientific Advisory Committee for the Oregon Regional Primate Research Center, Beaverton, and a member of the Personnel Committee of the American Cancer Society, New York. Dr. Eddy also served as a Discussion Leader at the Gordon Research Conference (GRC) on "Fertilization and Activation of Development" and as a Session Chairman at the International Testis Workshop held at the National Institutes of Health, Bethesda, Maryland.

Dr. McLachlan was the Vice Chairperson of the Gordon Research Conference on the "Mammalian Genital Tract" and served as Discussion Leader at this GRC. Dr. McLachlan was also appointed as a member of the organizing committee for the Gordon Research Conference on Hormonal Carcinogenesis to be held in 1985. In addition to these activities with the GRC, Dr. McLachlan was the co-organizer of the Lectures on Reproduction and Development which are jointly sponsored by the NIEHS and the Piedmont Reproduction Colloquium.

Dr. Negro-Vilar was appointed a member of the Toxicology Review Panel, Human Reproduction Program, World Health Organization, Geneva, Switzerland. He also served as President of the III Pan American Congress of Andrology and was appointed President of the Pan American Group of Andrologists, with the task of organizing the IV Pan American Congress of Andrology in 1985. In addition, Dr. Negro-Vilar was a member of the ByLaws Committee of the International Society of Andrology and elected as a member of that Society.

Dr. Pratt is Chairman of the Publications Committee for the Teratology Society and also on the Committee to Select an editor for Teratology. In addition, Dr. Pratt has been appointed to oversee the activities of the Public Affairs Committee of the Teratology Society.

INFORMATION EXCHANGE

Communication of basic and applied information vital to environmental health problems is aided by establishing mechanisms for information exchange and by assuming editorial responsibilities. LRDT scientists are frequently asked to review manuscripts for journals oriented toward Biochemistry, Development, Endocrinology, Pharmacology, Toxicology, and Teratology.

Dr. Korach is on the Editorial Board of Environmental Health Perspectives.

Drs. Korach and McLachlan serve as coeditors on the Target Organ Toxicity Monograph--Endocrine System.

Dr. McLachlan is on the Editorial Board of the International Journal for Biological Research in Pregnancy.

Dr. Negro-Vilar was appointed to the Editorial Board of Endocrinology. He also serves on the Editorial Board of Neuroendocrinologia Latinoamericana. In addition, Dr. Negro-Vilar was editor of the book, Male Reproduction and Fertility, published by Raven Press. He also is co-editor with Drs. Paulson, Lucena, and Martini of the book, Andrology: Fertility and Sterility, to be published by Academic Press.

Dr. Pratt is on the Editorial Boards of Differentiation; Teratogenesis, Carcinogenesis and Mutagenesis; Craniofacial Genetics and Developmental Biology; and Environmental Health Perspectives.

TRAINING PROGRAMS

Environmental health is a new and demanding research area that is undergoing rapid change and growth. Consequently, there is a growing need for training to ensure adequate numbers of qualified and dedicated researchers in environmental health research. The Laboratory of Reproductive and Developmental Toxicology recognizes this need and our scientists are encouraged to participate in a wide variety of training activities, including accepting adjunct appointments at nearby universities, supervising graduate student research, developing graduate courses in environmental health, and participating in the Fogarty International Center's Visiting Program.

Visiting Fellows are in postdoctoral training programs in the laboratories of Drs. Eddy, McLachlan, and Negro-Vilar. Dr. Korach has a Visiting Associate within his laboratory.

Dr. Eddy is an affiliate of the Population Research Center, University of North Carolina.

Dr. Eddy spoke in the Reproductive Physiology Graduate Seminar series at Duke University.

Dr. Korach is an affiliate of the Laboratory of Reproductive Biology, University of North Carolina. He is also a sponsor for an honor student from North Carolina State University.

Dr. McLachlan is a member of the UNC Cancer Center. Dr. McLachlan also serves as a research advisor to medical students from UNC and Duke as well as Reproductive Endocrine Fellows from Duke University Medical Center.

Laboratory scientists have also been active in the training of graduate students. Graduate students are working in the laboratories of Drs. McLachlan and Pratt. Dr. McLachlan is on the graduate committees of students at Duke, UNC and North Carolina State and serves as advisor and coadvisor, respectively, to graduate students at North Carolina State's Department of Zoology and UNC Department of Biochemistry.

Drs. McLachlan, Korach, and Pratt have lectured at UNC, Duke, and North Carolina State in areas of their research expertise.

Dr. Negro-Vilar is an affiliate of the Laboratories of Reproductive Biology at the University of North Carolina. He has also presented lectures at UNC and East Carolina University.

Dr. Pratt organized a graduate level course at the University of North Carolina entitled "Developmental Toxicology and Teratology." Drs. Pratt and McLachlan gave lectures in the Interdisciplinary Toxicology curriculum coordinated by Dr. Pratt.

Dr. Pratt is an Adjunct Professor at the University of North Carolina School of Medicine's Department of Anatomy and Cell Biology. He is also a preceptor for the NIGMS-NIH Pharmacology Research Associate Training Program.

Dr. Teng is an Adjunct Associate Professor at the North Carolina State University School of Veterinary Medicine, Department of Anatomy, Physiology and Radiology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70010-08 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of Normal and Abnormal Embryonic Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. M. Pratt	Head, Experimental Teratogenesis Section	LRDT NIEHS
Others:	G. K. Andrews	Senior Staff Fellow	LRDT NIEHS
	R. P. DiAugustine	Research Chemist	LRDT NIEHS
	R. I. Grove	Staff Fellow	LRDT NIEHS
	C. S. Kim	IPA	LRDT NIEHS
	K. S. Morgan	NIH Postdoctoral Fellow	LRDT NIEHS

COOPERATING UNITS *(if any)*
Department of Pediatrics
University of Washington, Seattle

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Experimental Teratogenesis

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	3.0	3.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this research project is to understand at the morphological, cellular and biochemical levels various aspects of normal and abnormal embryonic development, especially relating to craniofacial development. Glucocorticoids are teratogenic in vivo, and our results using whole rodent embryo culture demonstrate they exert a direct effect on the embryo; day 8 embryos cultured for 48 hrs develop heart and neural tube malformations whereas day 10 embryos develop cleft lip in culture after a 48 hr exposure to triamcinolone acetonide. Cultured day 10 mouse embryos become responsive to epidermal growth factor (EGF) after 48 hrs in culture, during which time the initial stages of secondary palate formation are occurring; future studies will ascertain the effect of other growth factors and hormones. Our results indicate that maternal EGF does not cross the mouse placenta although an embryonic form of EGF appears in the conceptus on day 12 of gestation; future work will determine the site(s) of synthesis of this EGF within the embryo. A cell culture system has been developed in order to examine growth and differentiation of isolated embryonic palatal epithelial cells and EGF is an absolute requirement; the effects of EGF are enhanced in the presence of cyclic AMP. The dioxin TCDD appears to induce cleft palate by altering the differentiation of the medial palatal epithelial cells. Glucocorticoids exert their cleft palate inducing effects by inhibiting palatal mesenchymal cell proliferation; immunocytochemical studies have localized glucocorticoids receptors mainly to the palatal mesenchyme. In palatal mesenchyme cell culture, one of the major effects produced by glucocorticoids associated with growth inhibition is an alteration in the turnover of plasma membrane phosphatidylinositol; future studies will examine the effect in greater detail. An established line of human embryonic palatal mesenchymal cells, along with a liver-derived metabolic activating system, has been used to develop a short-term growth-inhibition screening test for potential teratogens.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Normal development of the mammalian embryo is a series of extremely complex developmental events including cellular migration, proliferation, recognition, differentiation, and programmed cell death. The organogenesis phase of embryonic development is the most sensitive period to the adverse effects exerted by various chemicals and drugs (teratogens) which result in birth defects such as cleft palate. In order to understand how various teratogens interact with embryonic tissues to disrupt development, we are utilizing a multidisciplined approach to attack this problem. Studies are conducted using cell, organ, and whole embryo culture along with various methodologies including scanning and transmission electron microscopy, immunohistochemical localization, growth and biochemical analysis, and recombinant DNA technology. The major problem of interest to us is the manner in which various craniofacial tissues accomplish a number of difficult developmental events, the influence of various hormones and growth factors on these events, and the mechanism by which selected teratogens interfere with these events.

Objectives:

Using whole rodent embryos in culture, we would like to understand the complex hormonal and nutritional requirements necessary for normal development, especially that of the primary palate. Our hypothesis is that as the embryo matures, it becomes less dependent upon exogenously added growth factors and becomes capable of synthesizing its own growth factors, such as embryonic epidermal growth factor (EGF). We also are testing the hypothesis that glucocorticoids exert a direct effect on the growth of the mesenchymal cells in the primary palate in order for cleft lip to be induced by this teratogen in culture.

The mechanisms of normal and abnormal secondary palate development are of great interest to us. We hope to understand how glucocorticoids interfere with growth of the secondary palatal mesenchymal cells and are examining various biochemical parameters including receptor and cell surface protein localization and turnover of various phospholipids. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) appears to produce cleft palate by interfering with some aspects of terminal differentiation of the palatal medial epithelial cells. This hypothesis is being tested directly in organ and epithelial cell culture derived from the secondary palate. We plan to develop a short-term in vitro assay for screening teratogens using the endpoint of growth inhibition with our established line of human embryonic palatal mesenchymal cells.

Experimental Approach, Scientific Justification, and Recent Accomplishments:

(1) Normal and abnormal development of the mammalian secondary palate continues to be the major research interest of the Experimental Teratogenesis Section. Formation of the secondary palate involves a number of interesting

developmental phenomena including programmed epithelial cell death, epithelial-mesenchymal interactions, morphogenetic movements, and cellular adhesion and recognition. These events occur in the mouse on gestational days 12-14 and are accessible to a number of experimental manipulations including cell and organ culture. Glucocorticoids are rather specific inducers of cleft palate in many strains of mice and appear to directly affect the developing palatal shelves through a receptor-dependent process. Recent immunocytochemical studies, using polyclonal antibodies against the activated rat liver glucocorticoid receptor, have shown that the glucocorticoid receptors appear to be localized mainly in the palatal mesenchymal cells, and we feel these are the primary target cells for glucocorticoids with respect to cleft palate formation. Our cell culture studies using primary mouse mesenchyme and established HEPM demonstrate that glucocorticoids inhibit both mesenchymal cell proliferation as well as synthesis of extracellular matrix components including specific types of collagen and glycosaminoglycans. Both of these biochemical effects in vivo result in smaller sized palatal shelves which are unable to make contact and fuse. Future studies are aimed at determining the biochemical mechanism by which pharmacologic concentrations of glucocorticoids result in inhibition of mesenchymal cell growth. We also have studies in progress (see Andrews) to determine whether glucocorticoids play a role in α-fetoprotein and metallothionein gene expression in the developing mouse liver and yolk sac.

Epithelial cell growth and differentiation in the secondary palate results in three distinct phenotypes: the ciliated mucous-secreting nasal cells; the squamous, keratinizing oral cells and the medial epithelial cells which are programmed to die. One of our major research goals is to understand what factors influence the normal and abnormal development of these phenotypes. We have developed in vitro conditions under which the growth and differentiation of the isolated epithelial cells can be examined. The day 12 to 13 palatal epithelium is enzymatically removed from the mesenchyme and allowed to attach to an extracellular-matrix coated culture dish rich in type I and III collagen and fibronectin. Growth factor and hormonal requirements have been determined by culturing in a serum-free medium. Epidermal growth factor is an absolute requirement for adhesion, growth and differentiation of the epithelial cells, especially the oral cells which seem to be the most responsive cell type. This is due, in part, to the fact that the oral cells have the highest amount of EGF receptors, as demonstrated using labeled EGF and autoradiography. This effect of EGF on the oral cells can be greatly enhanced either by adding cyclic AMP or co-culturing on lethally-irradiated 3T3 embryonic fibroblasts. It appears that there is a stem cell population in the oral epithelium which, under the appropriate conditions, can grow for long periods in culture. In the presence of EGF, the medial epithelial cells do not undergo programmed cell death while the nasal cells do not appear to respond to EGF.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent cleft palate inducer in certain strains of mice. The strain sensitivity is related to the distribution of the Ah locus. The Ah locus determines the induction of several cytochrome P-450 mediated monooxygenase activities and the major gene product of this locus in the cytoplasmic receptor protein. Our results in CD-1 and C57BL/6J mice indicate that TCDD exerts a direct effect on the developing palatal shelves through a receptor dependent mechanism that results

in a lack of medial epithelial cell death. As a result, the palatal shelves, which are not inhibited in their growth, make contact in vivo but fail to fuse due to the TCDD-induced defect in cell death.

(2) Inositol-Containing Lipids. Recent evidence has linked the receptor-mediated degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) and/or phosphatidylinositol (PI) to the generation of intracellular secondary messengers thought to play a key role in regulation of cell proliferation. Glucocorticoids, which induce cleft palate in rodents by inhibiting palatal mesenchymal cell proliferation, markedly alter normal PI degradation and synthesis in a cell line derived from human embryonic palatal mesenchyme. This alteration in PI metabolism correlates in both dose response and time course to hormone-induced inhibition of cell proliferation. More recently, we have shown the hormone-induced alteration in PI metabolism to be receptor mediated using inactive hormone analogues. Furthermore, we have shown the alteration in PI metabolism to be independent of extracellular Ca^{++} concentration, intracellular Ca^{++}-Calmodulin complex formation, and intracellular cyclic AMP levels. The alteration in PI metabolism is exacerbated by lithium ion which is known to inhibit a key enzyme in the recycling of the inositol moiety into newly synthesized PI. This finding provides additional evidence that the hormone-induced alteration in PI metabolism is due to an increase in degradation of pre-existing PI. The correlation between glucocorticoid inhibited cell proliferation and alteration of PI metabolism has been strengthened by the finding that stimulators of proliferation which also stimulates the PI cycle (i.e. serum) are inhibited by glucocorticoids while stimulators of proliferation which do not stimulate PI metabolism (EGF) are not inhibited by glucocorticoids. Further investigation is directed toward determination of specific PI metabolizing enzymes that glucocorticoids may alter. In addition, the effect of glucocorticoids on PIP_2 levels are under current investigation. It is possible that glucocorticoids initially alter PIP_2 levels which result in a change in the equilibrium established between PI $\rightleftharpoons$ PIP $\rightleftharpoons$ PIP_2 and ultimately alter PI metabolism. The results of these investigations are expected to yield additional information on the mechanism by which glucocorticoids alter inositol-containing lipid metabolism.

(3) Glucocorticoids and Oncogene Expression. The expression of the cellular homologue (protooncogene) of various oncogenes has been linked to progression through the cell cycle. In addition, several polypeptide growth factors appear to stimulate tyrosine phosphorylation upon binding to their cell membrane receptors. These findings taken together with the fact that several oncogenes appear to code for tyrosine kinases, suggest that one way glucocorticoids may inhibit cell proliferation is via alteration of protooncogene tyrosine kinase expression. Recently, tyrosine kinases have been found to stimulate the phosphorylation of inositol-containing lipids (i.e. PI$\rightarrow$PIP$\rightarrow$$PIP_2$) which are thought to play a key role in regulation of cell proliferation. Since glucocorticoids alter both cell proliferation and PI metabolism, we are investigating effects of glucocorticoids on the expression of various oncogenes.

(4) Most teratogens exert their effects during the organogenesis phase of embryonic development which occurs from days 6 to 14 in the mouse. In vivo approaches to understanding the mechanisms of teratogenesis are useful, but

somewhat limited, since manipulation of certain variables, such as length and dose of teratogen exposure, is not possible. In vitro culture of the rodent embryo is now possible during days 8-12 of organogenesis when the embryo is undergoing its most dramatic growth and organ development. We have spent a considerable amount of time in determining the most appropriate culture conditions and medium for normal growth. Our results demonstrate that early somite (day 8) CD-1 mouse embryos cultured in 100% rat serum for 48 hours grow and develop to the equivalent of day 10 embryos in vivo, whereas day 10 embryos cultured for 48 hours do best in 50% fetal calf serum. To examine craniofacial development after day 12, which is one of our major research interests, we have found that embryonic heads can be cultured in the same roller bottle system for day 8 and 10 embryos using Dulbecco's modified MEM containing 10% fetal calf serum. Under these conditions, we have been able to obtain complete development of the primary (upper lip) and secondary palate (roof of the mouth); this has not been previously achieved by another lab.

With these culture systems, we have taken two major research directions. The first is to understand the complex hormonal influences on embryonic growth and development. The second is to understand the mechanism by which various craniofacial teratogens, such as glucocorticoids and TCDD affect embryonic development in culture. Glucocorticoids exert stage specific effects on embryonic development: heart and neural tube malformations are induced between days 8 and 10 in culture; cleft lip is observed between days 10 and 12. TCDD induces a high percentage of cleft palate when given on day 11 of gestation in the mouse and presumably affects some aspects of palatal epithelial development at this time. Previous studies in our lab have provided evidence for the production of an embryonic type of EGF starting on days 11-12 of gestation. We have also shown that adult EGF does not cross the placenta and, thus, the need for an embryo-derived EGF. Addition of EGF to cultured embryos prior to day 11 either has no effect or an inhibitory effect. Addition after day 11 appears to be growth stimulatory and corresponds to the acquisition of EGF receptors. Studies will be conducted in order to obtain optimal culture conditions with combinations of various hormones and growth factors such as glucocorticoids, insulin, transferrin, EGF and thyroxine. Ultimately, we hope to be able to culture the day 10-13 embryos and heads in a completely serum-free, hormone supplemented medium.

(5) Whole animal tests for teratogenesis are extremely expensive, time consuming, and inadequate to test all the chemicals now in existence and those introduced each year which may present a risk for the unborn. At the present time, there is a great deal of interest in many labs throughout the world to develop a short-term inexpensive in vitro assay that could predict those chemicals of greatest potential risk to the human population. These in vitro assays would not eliminate animal testing but would serve to indicate which of the thousands of chemicals that have not been tested should have the highest priority for in vivo testing. Our lab has developed a cell culture assay which should be useful to study teratogenesis. This assay is based on the sensitivity of a line of human embryonic palatal mesenchyme (HEPM) cells to chemically-induced growth inhibition over a four-day period in culture. Inhibition of growth of palatal mesenchyme cells by a number of diverse chemicals and drugs is the major mechanism by which cleft palate is induced in

the rodent, and the currently available evidence suggests that growth inhibition is the major etiology of human cleft palate. Therefore, these HEPM cells, established from a human embryonic palate at eight weeks of gestation, may be an ideal cell type to represent undifferentiated mesenchymal cells from a number of embryonic tissues which are frequently malformed in the human. To date, we have tested over 65 chemicals and drugs in which there is some information on the animal and/or human teratogenicity. There is a good correlation between the dose which causes a 50% inhibition of growth (ID_{50}) and the known drug and chemical teratogenicity. Chemicals and drugs which are poor or weak teratogens have either no effect or an ID_{50} of 1mM or greater, whereas those which are proven teratogens in vivo have an ID_{50} of 1 nM to 100 µM. The capability of an in vitro system to metabolize proteratogens, such as cyclophosphamide, to teratogens (phosphoramide mustard) is a highly desirable feature. We have spent considerable time developing conditions for such an activation system. HEPM cells are directly exposed to the arochlor-induced rat liver S-9 plus cofactors and teratogen for only 4 hours during the day after the cells are seeded onto the culture dishes. Under these conditions, we obtain an S-9 dependent dose-responsive inhibition of growth with cyclophosphamide (ID_{50} = 5 µg/ml). Dr. Thomas Shepard of the University of Washington is collaborating with us by sending the craniofacial regions from spontaneous and therapeutic abortions in order that additional lines of HEPM can be established for future use.

Significance:

Exposure to adverse environmental factors is presumably a major cause of developmental abnormalities in man. The laboratory prediction of teratogenic chemicals is unsure and the mechanisms of teratogenesis are unclear. An in vitro system for culturing embryos is providing an opportunity to study cellular and molecular processes associated with normal and abnormal development. Human embryonic cells are useful in predicting the toxic potential of environmental agents and in understanding the biological mechanism by which chemicals may disrupt early development and produce birth defects.

Facial clefting is a significant human congenital malformation and is one of the most frequently observed abnormalities. The etiology of facial clefting is complex, but appears to involve both genetic and environmental factors. The mouse is an excellent model since various inbred strains are highly sensitive to craniofacial teratogens. Our studies involve whole animal as well as cell and organ culture to further define the complex interactions between genetic and environmental factors. Information from our studies may lead to better genetic counseling and drug avoidance during pregnancy and may reduce the frequency of congenital facial clefting in the human.

B. PLANS FOR SUBSEQUENT YEAR

Whole embryo culture is an extremely powerful method to examine normal and abnormal embryonic development. The development of defined media has greatly increased our understanding of growth regulation in cell culture. The development of a low serum or defined media would also greatly enhance our understanding of embryonic development. In addition, studies with teratogens

such as glucocorticoids would be less complex due to the absence of serum components which may influence the action of various hormones.

The secondary palate appears to be an ideal tissue in which to understand the role of various growth factors such as EGF. EGF appears to have important effects on both palatal epithelial and mesenchymal cells, and our ability to culture these cell types in long-term culture will allow us to more clearly understand this dependency on EGF. We hope to localize the sites of EGF synthesis within the palate and critically test our hypothesis that EGF is an autocrine growth factor in palatal development. Recent in vivo studies demonstrate a synergistic interaction between TCDD and glucocorticoids with respect to cleft palate induction. Future studies in vivo as well as in culture will further define this synergism at the biochemical level.

We plan to develop monoclonal antibodies to various ligands on the surface of the palatal epithelial and mesenchymal cells. We hope to be able to utilize several of these monoclonal antibodies as specific probes for palatal development and plan to examine the effect of various agents including EGF, dexamethasone, and TCDD on their expression. Labeling studies in conjunction with one- and two-dimensional PAGE should enable us to identify the biochemical nature of these ligands. Addition of antibody to cell, organ and whole-embryo culture will enable us to ascertain the functional role of these ligands.

Recent evidence in a number of biological systems strongly suggests a role for the phospholipids, including phosphatidylinositol (PI), in transmitting signals across the cell membrane. Our evidence suggests that glucocorticoids may alter palatal mesenchymal cell growth by disrupting the PI cycle. Future studies will be conducted in order to further understand the role that the PI cycle plays in oncogene expression in embryonic cells as well as in normal palatal development and disruption by various teratogens.

PUBLICATIONS

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Kim, C.S., Lauder, J.M., Joh, T.H., and Pratt, R.M.: Immunocytochemical localization of glucocorticoid receptors in midgestation murine embryos and human embryonic cultured cells. J. Histochem. Cytochem. (In Press).

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Pratt, R.M., Perry, E.L., Chapman, L.M., and Goulding, E.H.: Glucocorticoid teratogenesis in mouse whole embryo culture. Teratology (In Press).

Silver, M.H., Murray, J.C., and Pratt, R.M.: EGF stimulates type V collagen synthesis in cultured murine palatal shelves. Differentiation (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70015-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Gene Expression During Murine Development

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: G. K. Andrews Senior Staff Fellow LRDT NIEHS

Others: R. M. Pratt Head, Experimental Teratogenesis Section LRDT NIEHS

COOPERATING UNITS *(if any)*
La Jolla Cancer Research Foundation, La Jolla, California

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Experimental Teratogenesis Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was designed to study molecular events which may regulate gene expression during development. The work to date has focused primarily on expression of the murine alphafetoprotein (AFP) and metallothionein (MT) genes with particular emphasis on studying effects of glucocorticoids on mRNA levels in developing mice. Both AFP and MT genes are actively expressed in visceral yolk sac endoderm as well as in fetal and neonatal liver. We have found that in neonatal liver, glucocorticoids can induce a 3-5 fold decrease in AFP mRNA with a concomitant 5-10 fold increase in MT mRNA. However, during fetal development, glucocorticoids, even at teratogenic levels, did not result in alterations in AFP or MT mRNA levels in visceral yolk sac. Furthermore, hepatic AFP mRNA levels were unaffected by glucocorticoid and only after day 16 of gestation was there a small glucocorticoid-induced increase in hepatic MT mRNA. Since the actions of steroid hormones are thought to be mediated by specific receptor molecules in the target tissue, we analyzed developing visceral yolk sac and liver for glucocorticoid receptors. There was an 8-fold increase in receptor levels in visceral yolk sac (i.e. to 10,000 sites/cell) between days 9 and 14 of gestation. Receptor levels in fetal liver were constant (2-4 x 10^3 sites/cell) until day 16 and increased slightly to parturition. Hepatic receptor levels increased rapidly after birth reaching adult levels (i.e. 30,000 sites/ cell) by days 10-14 post-partum. These results indicate that receptor levels may be limiting the glucocorticoid responses in early stages of fetal liver development, whereas, in visceral yolk sac, presence of receptor alone is not sufficient for response, at least as measured by changes in AFP and MT mRNA levels. Heavy metals (i.e. Cd^{+2}, Zn^{+2}, Cu^{+2}) were found to induce an increase in MT mRNA in neonatal liver without affecting AFP gene expression.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Cell specific regulation of gene expression is thought to be the major mechanism by which differentiation occurs. Thus, understanding events which regulate or alter normal patterns of gene expression can provide clues as to the mechanisms of both normal and abnormal development. The teratogenic effects of glucocorticoids as well as heavy metals are well documented; however, little is known about molecular mechanisms of action of these substances in the embryo and fetus. The studies proposed herein will attempt to elucidate the events involved in regulation of the AFP and MT genes in fetal and embryonic mice.

Objectives:

The overall goal of these studies is to understand the mechanisms of regulation of gene expression during development. The specific near term objectives of this project are (1) to determine the ontogeny and tissue specificity of expression of the AFP and MT genes, (2) to analyze the effects of glucocorticoids and metal ions on expression of these genes during development, and (3) to identify putative regulatory DNA sequences or regions which may regulate glucocorticoid responsiveness of the AFP gene.

Experimental Approach and Scientific Justification:

Glucocorticoid hormones have been shown to increase hepatic MT mRNA levels in adult mice. Conversely, glucocorticoids cause a decrease in hepatic AFP mRNA in neonatal mice. This provides an attractive system in which to analyze hormone effects on hepatic gene expression during development. Heavy metals have also been shown to induce hepatic MT mRNA in the adult, although nothing is known about the effects of metal on AFP gene expression.

The initial approach taken in this work has been to analyze the tissue specificity and temporal expression of the AFP and MT genes during embryonic fetal and neonatal development. Essentially nothing was known about these aspects of MT gene expression. Expression of these genes was quantitated by measuring mRNA levels via dot blot and Northern blot hybridization using cloned cDNA probes. After the normal developmental pattern of expression of AFP and MT is clarified (see below), we are attempting to perturb expression of these genes by injection of teratogenic doses of glucocorticoids or metal ions. In conjunction with the above work, we are correlating expression of these genes at the mRNA level with relative rates of transcription of these genes as measured by using polymerase run-off experiments. In addition, we are developing assay protocols which will utilize the purified AFP gene to identify regulatory DNA sequences involved in the hormone responsiveness of this gene. This is being approached by DNA transfection experiments coupled with *in vitro* hormone receptor binding assays.

An embryonal carcinoma cell line F9 derived from a mouse teratocarcinoma (germ cell tumor) can be induced to differentiate into two distinct populations of extraembryonic cell types, parietal and visceral endoderm. F9 monolayers treated with retinoic acid (Vitamin A) and dibutyryl cyclic adenosine monophosphate differentiate into an early embryonic cell type parietal endoderm. F9 cells treated with retinoic acid in suspension culture differentiate into aggregates called embryoid bodies. Embryoid bodies are so-called because they morphologically resemble early mouse embryos at the two-layered stage. Embryoid bodies synthesize large amounts of alphafetoprotein, transferrin, and metallothionein (a recent finding of this lab), which is characteristic of visceral yolk sac endoderm. This cell culture system is being exploited to analyze molecular mechanisms affecting expression of visceral endoderm specific genes. This work is being carried out in collaboration with Dr. E. D. Adamson of the La Jolla Cancer Research Foundation.

These above approaches will provide important information regarding mechanisms which may regulate gene expression during normal and abnormal embryonic development.

Recent Accomplishments and Significance:

Since the inception of this project in the spring of 1983, several important advances have been made. The ontogeny and tissue specificity of expression of MT in mice was described and compared with previous data on AFP. The MT gene was found to be expressed in high levels in endoderm of visceral and parietal yolk sacs. This indicates that the yolk sac may be the primary site of teratogenic action of metal ions. Visceral endoderm also synthesizes AFP and transferrin, and, thus, disruption of yolk sac function due to metal accumulation would have profound effects on fetal development. Synthesis of MT and AFP was shown to occur later in gestation in fetal hepatocytes. Since expression of AFP and MT can be influenced by glucocorticoids, it was of interest to determine the effects of glucocorticoids on mRNA levels in fetal tissues. We recently found that AFP and MT mRNA levels can be dramatically affected in neonatal liver but apparently not in fetal liver or yolk sac, even with teratogenic doses of hormone. This indicates that the glucocorticoids are not responsible for induction or modulation of expression of MT or AFP genes during embryonic development. Since glucocorticoid action is a receptor mediated event, it was of interest to determine the ontogeny of glucocorticoid receptors in yolk sac and liver. We recently obtained the following results: (1) the visceral yolk sac was found to contain glucocorticoid receptors, and changes during gestation were described; and (2) the fetal liver contained only low levels of receptor which increased dramatically after birth. The presence of glucocorticoid receptor in the yolk sac has not been previously reported, and the ontogeny of these receptors in liver was unknown. Our results are consistent with the hypothesis that glucocorticoids do not induce MT or AFP gene expression in fetal liver or visceral yolk sac. The presence of high receptor levels in visceral yolk sac during late gestation implies a role for glucocorticoids in yolk sac development which was hitherto unreported.

B. PLANS FOR SUBSEQUENT YEAR

In the next year or two, this project will examine the following aspects of this problem. The effects of metal ions on developmental expression of MT and AFP genes will be explored. Results of these studies will provide information on the involvement of metal in the normal expression of fetal genes. In addition, we will be able to determine whether metal ions can directly influence gene expression in the visceral yolk sac and liver. Hormone and metal ion effects on the relative rate of transcription of AFP and MT genes will be studied. These experiments will allow us to ascertain the level at which these effectors may modulate expression of these genes. Whether glucocorticoids will affect the relative rate of transcription of the AFP gene is unknown. We will examine this in detail in the neonatal liver. We also plan to develop further a project which will allow for identification of putative regulatory DNA regions for the AFP gene. The 5'-flanking DNA sequences of hormone responsive genes are involved in binding of hormone-receptor complexes and RNA polymerase II. These binding events may regulate qualitatively and quantitatively the transcriptional activity of many hormone responsive genes. The 5'-flanking DNA of the AFP gene will be used to study binding of glucocorticoid-receptor complexes in vitro. Sequence analysis of this region coupled with deletion mutagenesis may allow for mapping of putative control regions for the AFP gene. Cultured cells can be transfected with foreign DNA. This DNA can be integrated into chromosomes and expressed by the recipient cell. The 5'-flanking DNA sequences of the AFP gene have been fused with the HSV-thymidine kinase gene and will be transfected into a TK deficient mouse cell line which contains glucocorticoid receptors. This system will be used to examine the involvement of specific sequences of the alphafetoprotein gene in response to glucocorticoid in vivo.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70060-11 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 chereters or less. Title must fit on one line between the borders.)*
Developmental Biology/Toxicology of Estrogenic Environmental Chemicals

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. McLachlan	Head, Devel. Endo. and Pharm. Section	LRDT NIEHS
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	K. S. Korach	Research Endocrinologist	LRDT NIEHS
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	J. C. Barrett	Research Biologist	LPFT NIEHS
	R. P. DiAugustine	Research Chemist	LRDT NIEHS
	C. T. Teng	Expert	LRDT NIEHS

COOPERATING UNITS *(if any)*
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Duke University Medical Center
Medical Foundation of Buffalo
University of Würzburg
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LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Developmental Endocrinology and Pharmacology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.6	2.8	2.8

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues x☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Studies have continued to determine the molecular forms and cellular targets of estrogenic chemicals and establish the mechanisms by which interactions of estrogens with developing genital tract target cells result in permanently altered differentiation, including neoplasia. In the period covered by the report, the ovary and oviduct have been clearly established as targets for diethylstilbestrol (DES)-induced dysmorphogenesis. The cellular alterations in the ovary are associated with functional defects--altered steroidgenesis. The epithelial cells of the oviduct no longer maintain appropriate growth controls. Experiments in vitro with the fetal anlage of reproductive tract tissues, the Müllerian duct, have demonstrated that the fetal tract is imprinted by DES at the molecular level and becomes unresponsive to its normal tissue effector, Müllerian Inhibiting Factor. Futhermore the fetal genital tract is capable of oxidatively metabolizing DES along pathways which generate reactive intermediates. This may play a role in the long-term dysdifferentiation of those target tissues, since DES metabolism has been also associated with genetic alteration in model cells in culture (Syrian hamster embryo fibroblasts) including aneuploidy induction and unscheduled DNA synthesis. These occurred under conditions associated with neoplastic transformation of cells in vitro.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

To determine the molecular forms and cellular targets of estrogenic chemicals and establish the mechanisms by which interactions of estrogens with developing genital tract target cells result in permanently altered differentiation, including neoplasia.

Objectives:

The work in this project is based on two central premises: That estrogens play an important role in genital tract differentiation and that exposure to estrogens at inappropriate times or amounts will permanently alter the developmental program of these tissues such that they respond atypically to further stimuli or cues. Furthermore, the hypothesis that metabolic conversion of estrogenic chemicals plays a role in their long-term toxicities (e.g., neoplasia) is being tested. The interplay of the hormonal activity of estrogens as stimulants of target cell proliferation with their pharmacologic properties in the final expression of neoplasia and dysmorphology is also a major focus of this research. For example, an important issue in hormonal carcinogenesis is the relative roles of cell proliferation or transformation in the induction of tumors. This issue underlies rational design of estrogenic drugs, as well as therapy and prevention of breast and gynecologic cancers. Thus, studies using in vitro cell culture systems in which the effects of hormones on cell division and differentiation may be distinguished from drug metabolism and the process of neoplastic transformation provides important new insights into the actual mechanisms whereby hormonally associated cancers arise. The further ability to determine chemical structures associated with cell proliferation and those with neoplastic transformation in isolated in vitro systems provides a powerful tool for the study of target cell biology as well as hormonal pharmacology with ultimate elucidation of rational structure-activity relationships in the normal and abnormal effects of hormones.

Experimental Approach, Scientific Justification, Recent Accomplishments and Significance:

The synthetic estrogen, diethylstilbestrol (DES), is a common environmental chemical currently used as a livestock growth promotor and gynecologic medication. Experiments in our laboratory have demonstrated that prenatal exposure to DES adversely affects the reproductive capacity of the male and female offspring. Continuation of these studies has shown that such prenatal exposure results in a low incidence of female genital tract neoplasms including vaginal and uterine adenocarcinoma, squamous cell tumors of the vagina and ovarian tumors. A common non-neoplastic lesion of the DES-treated mouse was squamous metaplasia of the uterus. This abnormality was determined to require a secondary stimulus by estrogen at puberty for its manifestation. Cellular changes suggest the term squamous cell transformation may be more appropriate since the

uterine derived squamous cells express many of the characteristics of estrogen stimulated vaginal squamous cells including estrogen receptor levels, cell turnover kinetics, secretory function, morphology and hormone dependence. Anatomical changes such as cervical enlargement without luminal size changes, altered utero-tubal junctions, and uterine shape changes may be important to understanding reported subfertility in similarly exposed women. Alterations in the surface morphology of the DES-treated mouse genital tracts were revealed by scanning electron microscopy. Stromal hyperplasia of the vagina, cervix and uterus has raised the question of the role of this tissue component in the observed lesions; the demonstration of uterine and cervical stromal sarcomas in prenatally DES-treated mice further emphasizes the importance of studies on stromal-epithelial interactions during abnormal development of the genital tract. Similar lesions could not be produced following prenatal exposure to the steroidal estrogen, 17β-estradiol. Differential fetal protein binding of DES and estradiol may help explain these results. Bioavailability at many levels may be a determining factor in the transplacental toxicity of hormonally-active xenobiotics. Moreover, the stage of cell differentiation at the time of DES exposure is critical in determining the type of lesion expressed later in life. DES treatment during early cytodifferentiation of the female genital tract is associated with vaginal adenocarcinoma but very little vaginal adenosis; treatment during later cytodifferentiation of this tissue is associated with adenosis but not vaginal adenocarcinoma. Ovarian abnormalities in morphology and steroid secretion in prenatally DES-treated mice appear to arise from altered differentiation of the fetal ducts which contribute to ovarian morphogenesis.

The control of cell differentiation and proliferation in the genital tract is obviously a central issue to our studies. This will be further studied utilizing mesenchymal and epithelial cell cultures. Thus a collagen-gel cell culture system utilizing epithelial cells from mouse seminal vesicle (Wolffian derivative) and uterus (Müllerian derivative) has been established in defined media; under these conditions cells will respond to hormones by expression of appropriate secretory proteins or proliferation. Moreover, the role of genital tract derived growth factors, including epidermal growth factor (EGF), in the proliferation and development of the uterus will be determined. Alteration of these processes by early treatment with hormones will add insight to their importance. Preliminary results suggest that, at least in the case of the uterus, EGF may function as an estrogen stimulated autocrine growth factor.

In continuation of studies with male offspring from DES treated mice, the fate of Müllerian duct remnants in DES treated males was determined and the role of these tissues in prostatic lesions evaluated. These remnants were shown to respond to estrogen with typical Müllerian features. Müllerian remnants were observed in treated male offspring at all ages studied. Alterations in these tissues were, in part, related to changes in estradiol and Müllerian inhibiting factor (MIF). Most abnormalities observed in the genital tracts of exposed men and women have been duplicated in our mouse model. Additional alterations seen in the mouse may be predictive for the human. For example, testicular cancers, including seminomas, have been recently found in both mice and humans. In both male and female offspring, attempts will be made to establish biochemical/morphological markers for genital tract lesions. Gene probes for Wolffian duct

function and Müllerian duct function will be established and used to study the cell-cell interactions necessary for normal genital tract development. For example, protein profiles determined by SDS gel electrophoresis of the secretions of the prostate and seminal vesicles of DES mice were shown to be altered; castrate males exposed prenatally to DES and postnatally to estradiol had seminal vesicle secretory patterns identical to uterine luminal fluid. Seminal vesicle epithelial cells will be established in cell culture in defined media and response to hormones studied using genetic techniques and morphology. This model system will be used to study the underlying molecular and biochemical alterations which accompany aberrant differentiation.

The distribution, metabolism and structure-activity relationships of DES in perinatal systems have continued. Oxidative metabolites of DES (e.g. Z,Z-dienestrol and ω-hydroxy DES) were identified in the mouse fetus and neonate exposed to ^{14}C-DES. Recently, organ cultures of the fetal mouse genital tract have been shown to oxidatively metabolize DES. These findings are consistent with peroxidase mediated metabolism in the fetal target tissue. Moreover, a new metabolite monomethylated DES suggests another important pathway for DES metabolism which is observed only in target tissue (in vivo or in vitro). Studies on the bioactivation of DES have shown the non-extractable binding of radioactivity to DNA and protein after incubation of ^{14}C-DES with several activating systems including one derived from target tissue, the mouse uterus. DES was shown to be oxidatively metabolized during prostaglandin synthesis by the peroxidase activity of prostaglandin synthetase from sheep seminal vesicle microsomes. The role of this activity in target organ bioactivation remains to be established. The estrogenic activities of a series of DES metabolites and analogs were determined. Results suggest that DES metabolism follows alternative pathways resulting in metabolites which retain estrogenicity or those in which such activity is absent. These studies have been expanded with special emphasis on the biological significance of potentially activated metabolites; such data should aid in generalization to other classes of estrogenic environmental chemicals. In addition, studies of DES metabolism in target/non-target tissues and in cell culture are being continued. For example, it was shown that peroxidase, an inducible enzyme in estrogen target tissue, is able to metabolize DES to its major metabolite, Z,Z-dienestrol. Fluorinated derivatives of DES have been made to help assess the role of metabolism in toxicity. These studies are augmented by experiments on the in vitro metabolism of DES by a transformable cell system in which conversion of DES to Z,Z-dienestrol was demonstrated. Further studies suggest that metabolism of DES by these cells is mediated, at least in part, by cooxidation with prostaglandin synthetase. Moreover, DES and some of its structural analogs neoplastically transform these cells in vitro in the absence of measurable somatic mutation or stimulated cell proliferation. A better correlation was established for the peroxidative metabolism of the compound and cell transformation than its estrogenicity. Moreover, a cellular lesion associated with DES-induced cell transformation in vitro or in vivo is the induction of aneuploidy. The relationship of this chromosomal change to DES-induced neoplasia is being explored. Additional studies using steroidal estrogens and their catechol derivatives have demonstrated that the pharmacological principles established for neoplastic cell transformation and drug metabolism pertinent

for DES appear to apply to this class of "natural" estrogens. Studies using X-ray crystallography as well as computational models will be used to further explore the structural basis for estrogen-induced cell dysmorphogenesis.

Studies in organ culture have shown that DES can alter normal differentiation of the genital tract in vitro. These studies will be continued to evaluate the role of organ/organ and cell/cell interactions in genital tract development. Thus, DES has been shown to alter the action of MIF on the in vitro differentiation of the Müllerian ducts. These studies have raised the question of what biochemical changes are exerted by DES in the developing fetal Müllerian duct which stabilizes it against the regressing action of MIF. Two-dimensional gel electrophoretic maps of the protein changes during organogenesis of the female genital tract have been developed to aid in an understanding of the molecular events which determine normal or abnormal differentiation of this system. Currently, a single gene product (70K/5.8 pI) appears to be consistently and permanently deleted in dysmorphogenic genital tracts in DES treated mice. Also, experiments utilizing the separation and recombination of stroma and epithelium of DES treated fetal reproductive tracts have been undertaken to determine the role of such tissue interactions in DES induced genital abnormalities. Studies are being done to evaluate the role of cell proliferation in the fetal genital tract by different DES analogs in their transplacental carcinogenic potential. A new model in which a progressive proliferative lesion of the oviduct leading ultimately to uncontrolled oviductal epithelial proliferation with an altered differentiation program is being used to study control of Müllerian-derived epithelial proliferation and differentiation. This model and the dysmorphogenic dysfunctional ovarian model are helping solve the role of tissue interactions (Wolffian/Müllerian) during genital tract development. Finally mathematical models will be developed to test the fit of laboratory animal data in risk assessment for humans.

B. PLANS FOR SUBSEQUENT YEAR

(1) The mechanisms of DES-induced genital tract dysmorphogenesis will be studied in mice utilizing gene probes to the embryonic rudiments of these tissues--the Wolffian and Müllerian ducts; the probe for the Müllerian duct derivative will utilize the estrogen dependent uterine 70K secretory protein; that for the Wolffian will utilize the androgen dependent seminal vesicle secretory protein (SVS IV). The probes will be further utilized to study hormone-induced differentiation/proliferation of epithelial cells in collagen-gel cultures derived from these two tissue types (uterus/oviduct and seminal vesicle) as well as organ cultures of fetal genital tissues. Permanently expressed misprogramming in genital tract differentiation will also include studies on the expression and utilization of peptide growth factors. These studies will be underlaid by characterization of in situ models for regulation of cell proliferation and differentiated functions (e.g., secretion) following developmental perturbation with estrogenic compounds. The mechanisms whereby secondary estrogen exposures stimulate expression of cellular lesions will be studied in animal models.

(2) Structure-activity-relationships for estrogen-induced dysmorphogenesis (including neoplastic transformation) in vivo and in vitro will be further

characterized and DES and steroidal estrogens will be studied in genital tract organ cultures and cell cultures; special emphasis will be placed on peroxidative (including PES) and methylation pathways including their hormonal control and intraorgan/subcellular localization. Binding of estrogens to non-classical intracellular targets (i.e. tubulin, nuclear matrix) will be studied. Experiments to define non-receptor mediated effects of estrogens on target cells will be undertaken; these effects include neoplastic transformation and prostaglandin biosynthesis.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 ES 70065-08 LRDT

PERIOD COVERED

October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical-Receptor Interactions in Reproduction and Hormonal Toxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others:	J. A. McLachlan	Head, Develop. Endo. and Pharm. Section	LRDT NIEHS
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Burroughs Wellcome Research Labs
University of Wisconsin (Madison)
Duke University

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SECTION

Developmental Endocrinology and Pharmacology Section

INSTITUTE AND LOCATION

NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	1.3	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

DES, a potent synthetic estrogen and reproductive toxicant has been shown to be extensively metabolized. Some metabolites retain hormonal activity while others are biologically inactive. This assignment of activity is consistent with the receptor binding activity of these compounds. Besides reproductive tract effects some of these metabolites also elicit neuroendocrine effects by suppressing LH secretion. Two groups of metabolites were found to have poor uterotropic activity although they bound very well to the receptor. Some of these compounds show a variety of differences compared to the intracellular responses of DES, including lack of receptor synthesis, poor nuclear translocation, excessive retention of the receptor complex in the nucleus and the inability to stimulate certain tissue responses.

Estrogen stimulation of reproductive tract tissue involves a mechanism which includes binding to a receptor with subsequent activation and localization in the nucleus. Nuclear translocation follows a biomodal temporal pattern consistent with the stimulation of certain tissue responses. Only biologically active compounds induce these receptor events. The two receptor events appear to be occurring in different uterine cell types with uptake in the stroma first followed by the epithelium. Cell cycle kinetic studies of uterine estrogen stimulation show that the second peak occurs at the beginning of S-phase. A major effect of estrogen on uterine cells was to shorten the cell cycle by contracting the G1 phase.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Exposure to environmental agents produces a variety of effects on the reproductive tract, some which result in infertility and toxicity. In many cases such effects are produced by agents which have estrogenic hormonal activity but varying chemical structures. In order to better understand such differences, studies are underway to determine the structural and chemical basis for estrogenic responses. Estrogens mediate their actions via a receptor at the target site. However, the exact biochemical role of these receptor macromolecules and their intracellular mechanism of action is not well understood; since this applies to the endogenous ligand (estradiol), the problem is compounded by studying environmental xenobiotics. Therefore, research is required to characterize the biochemical reactions of the estrogen and progesterone receptors in modulating these processes.

Objectives:

Structure-activity studies using a variety of DES metabolites and analogs are being performed to assess the estrogen receptor binding activity as well as their ability to localize in the target cell nucleus and induce hormonal responses. These compounds are an interesting series since they vary structurally and chemically around the common chemical nucleus of DES. Most interestingly, small variations in structure result in significant differences in hormonal activity and receptor binding affinity. By using this comparative approach, questions concerning the relationship of ligand binding to the activation state and the genomic interaction of the receptor complex can be answered. It should also be possible to determine if these different complexes induce hormonal responses differentially and individually or in concert. This would gain insight into separating the early hormonal responses from the later actions involving uterine growth. Besides investigating DES compounds, a series of polychlorinated biphenyl (PCB) compounds with different chlorinated substitutions are being assayed for receptor binding and uterotropic stimulation. PCB's have been shown to cause reproductive toxicity, but the mechanism is not well understood mainly because these compounds exist as a complex mixture and testing of the hormonal activity of individual isomers has not been done. By synthesizing various PCB derivatives and measuring the activities, it should be possible to determine what chlorinated substitutions are influential in hormonal activity. It is planned that by using these simple chemical structures in conjunction with the more complex DES compounds a knowledge will be gained of the molecular structure involved in receptor binding and hormonal responses.

In order for a chemical to induce uterotropic response, it must interact with the estrogen receptor and initiate a series of events culminating in uterine growth. At present, it is thought that after the initial receptor binding the ligand receptor complex localizes within the nucleus and interacts with various

genomic sites to induce tissue responses. The primary responses are increased gene transcription and ultimately hyperplasia. From past studies, it has been suggested that all those responses are stimulated in concert but temporally at different times. It is not well understood if the concentration of nuclear receptor at certain times is critical to eliciting the responses or whether a minor population of receptor complex at specific gene sites is necessary. This is being investigated by determining the temporal pattern of estrogen receptor distribution during stimulation. Concomitantly, the temporal pattern of DNA synthesis and increases in DNA polymerase activities will be determined. Other responses such as enzyme induction of glucose-6-phosphate dehydrogenase, ornithine decarboxylase and progesterone receptor will also be investigated. Since estrogens are mitogens in the uterus, it is of interest to evaluate if growth factors are involved in this estrogen action. This will be approached by studying the role of the estradiol receptor in the mitogenic action of estrogens and EGF in uterine epithelial cells in culture.

Experimental Approach, Scientific Justification, Recent Accomplishments, and Significance:

DES is a potent synthetic estrogen which also undergoes extensive oxidative metabolism. In order to determine whether the metabolism of DES resulted in hormonally active or inactive metabolites, certain DES metabolites and analogs were tested for estrogenic activity using both an in vivo uterine bioassay and an in vitro receptor binding assay. Results of these studies showed good correlation between the biochemical and bioassay data. Compounds such as DES epoxide and dihydroxy DES as well as the catechol DES compounds but not their methylated forms showed reasonable receptor binding and biological activity while certain metabolites, e.g., ZZ-dienestrol or ω-hydroxy dienestrol, showed weak receptor interactions and poor estrogenicity. This indicates that the metabolism of DES along certain pathways does not result in complete hormonal inactivation. In the reproductive tract, DES has been shown to stimulate uterine peroxidase enzyme activity. Interestingly, DES is a metabolic substrate for this enzyme system which produces a DES p-quinone intermediate which rearranges to a Z,Z-dienestrol product. Other studies have suggested that DES quinone is a highly reactive compound which interacts irreversibly with DNA and protein. Therefore, it was interesting to determine if the quinone would interact with the receptor. Results using competitive binding assays and rate inhibition studies indicate that the quinone binds to the estrogen receptor. Further studies are planned using radioactively labeled quinone to determine if irreversible binding is present. Exceptions to the agreement between receptor binding and biological activity were seen with two groups of DES metabolites, indenestrol isomers and the ψ DES isomers, which show receptor binding comparable to DES, but were 20-100 times less biologically active. Compounds in these groups have subtle structural differences which differ by positions of a double bond or orientation of a methyl group. The E-ψ isomer has significantly lower activity than the Z-ψ isomer as evidenced by induction of progesterone receptor, uterine growth, and DNA synthesis studies. Receptor binding was 20 times weaker for the E-isomer that the Z-form. In order to establish if the receptor binding and biological activity

were due to structural differences in the two isomers, x-ray crystallographic studies comparing their molecular structures were performed. Both isomers showed a similar crystal structure with the rings oriented at right angles compared to the extended planar ring structure of DES. Other steps in the estrogen receptor mechanisms have also been investigated and indicate that the E-isomer does not translocate or retain receptor in the nucleus as effectively as the Z-isomer. Characterization of the pseudo DES and indenestrol nuclear receptor interactions will be continued to determine if significant differences can be detected to explain their altered biological activity. Studies using the DES metabolites and analogs are being expanded to include investigation of their neuroendocrine activity. Preliminary results indicate that the indenestrol isomers show differences in their ability to suppress LH secretion. Testing of other compounds will be performed to determine if the same degree of biological activity exists at this in vivo estrogenic target site.

Studies of receptor and plasma binding activities, particularly to alpha fetoprotein, of various DES analogs and metabolites will be continued to determine the structural basis for chemicals exhibiting hormonal and/or carcinogenic actions. These structural requirements were exemplified from studies determining the estrogen mitogenic activity of the DES compounds. Two of the indenestrol isomers, differing only in the position of a double bond, showed divergent uterine DNA stimulation. Another series of hydroxylated ψ-DES isomers has indicated that receptor binding of these ligands can be diminished by subtle structural differences. Chlorinated pesticides o,p DDT, o,p DDE and certain hydroxylated metabolites and isomers are being tested for receptor binding properties and hormonal activity. These compounds show measurable interactions but are weakly estrogenic. This does not preclude the possibility that repeated continued exposure could stimulate estrogen responses. Additional studies are planned to study the estrogenicity of these compounds using pellet implants simulating long-term low dose exposure. Another series of structural studies are investigating the binding properties of isomeric forms of polychlorinated biphenyls (PCB). These compounds have some reproductive toxicity which may be due to inherent hormonal activity. Results have shown that certain isomers such as 2,4,6-Trichloro,4'-hydroxybiphenyl have reasonable receptor binding activity which can be increased by positioning of the chlorine substitutes in specific spacial orientations relative to estradiol. Experiments testing the in vivo hormonal activity of these compounds will be planned once certain chemical isomers are identified from receptor binding studies.

Binding studies are being expanded to allow the potential hormonal activity of selected environmental chemicals to be determined using this model. A unique DES derivative containing fluorine atoms has been synthesized and its hormonal activity is being investigated. Preliminary studies indicate this compound interacts with receptor binding sites in a biphasic mechanism and that this complex can translocate to uterine cell nuclei. The derivative also appears to be very effective at stimulating uterine growth and DNA synthesis. Tests with this compound and other structural isomers using additional biochemical assays are underway.

In order to understand the tissue responsiveness to estrogens in more detail we investigated the mitogenic action in different uterine cell types. Studies indicated that estradiol induces DNA synthesis and mitosis in both uterine stroma and epithelium of sexual immature animals while mature adult animals show the mitogenic response only in the epithelium. Loss of stromal mitogenesis occurs between day 28-35 of development and coincides with sexual maturity. Animals which were ovariectomized on day 16 did not show the development of stromal responsiveness but rather gave an adult pattern. These experiments indicate that this tissue responsiveness may be influenced by the ovary. Studies will continue in order to determine and identify what ovarian substances may be involved in this effect. The mitogenic action of estrogen was similar in either effective cell type and was shown by thymidine autoradiography and fraction labeled mitosis (FLM) studies to be due to a contraction of the length of the uterine cell cycle. This shortening of the cell cycle was most significant in G1 phase with a slight effect during S-phase. Cell cycle studies also illustrated that the second nuclear receptor translocation event was occurring at the beginning of S-phase. Epidermal growth factor (EGF) receptors are being assessed in uterine tissue to determine if EGF may play a role in the mechanism of estrogen uterine mitogenesis. Early results indicate specific EGF receptor binding is present in uterine tissue. Further studies to determine the exact cellular localization of these EGF receptors and whether estrogens influence their expression or whether EGF plays a role in estrogen receptor activation are underway.

To study abnormal tissue responses to estrogens, mice treated developmentally with estrogens were used since the uteri are functionally altered in treated mice. Prenatal administration of DES results in female offspring of two distinct groups: those with hormonally nonresponsive uteri and those that are hyperstimulated. To understand why some uteri were not hormonally responsive, the concentration of estrogen receptors in these animals was examined. Results have shown that those animals in the nonresponsive group have significantly lower levels of estrogen receptor. In order to distinguish the age at which this difference in receptor level occurs between DES treated and control animals, receptor levels in 1 to 12 month old animals have been measured. These studies suggest that at 1 month of age there is no pattern of receptor differences. By 2-4, months differences in receptor levels between control and treated groups are noticeable and by 6 months, significantly lower levels are seen in the DES group. The differences in cytosol receptor levels seen after 4-6 months could not be explained by differential accumulation of receptors in the nucleus since assays of nuclear receptor in these same tissues showed no appreciable differences. Biochemical characterization of the receptor in the DES group showed similar molecular properties of binding affinity and sedimentation values and nuclear translocation capacity as controls. Endocrine manipulation using ovariectomy and adrenalectomy can result in an animal with similarly lower uterine receptor levels as the DES animal. However, hormone responsiveness in these animals is not compromised as it is in DES exposed animals. Uterine progesterone receptor is an estrogen specific response which can be induced in ovx/adx animals but not in the DES treated group. In order to establish how early in development the receptor system is present, measurements or receptor levels in fetal reproductive organs are being planned. Due to the limited amount of fetal tissue a micro steroid receptor assay using

^{125}I-estradiol and estrogen receptor monoclonal antibody will be developed. The ^{125}I-estradiol binding assay is advantageous since it has a higher ligand specific activity than tritium labeled steroid. The receptor antibody assay will detect receptor protein independent of the receptor ligand binding activity. Individually or in combination, these assays should increase our ability to detect low levels of receptor. Tissue differences in cytosol receptor concentrations were demonstrated only in the uterus since vaginal tissue levels were not significantly different from controls. Further experiments with the uterus and vagina from DES exposed mice will determine which step in the mechanism of hormone action and which responses may be altered.

Receptor differences in control and DES treated offspring were also found in studies demonstrating the responsiveness of the receptors to estrogen administration. Initial studies in the mouse uterus indicated two estrogen receptor complex translocations to the nucleus after exposure to hormone. The first at 1 hr and the second at approximately 7-8 hr. The relationship of this second receptor event to tissue responsiveness was demonstrated by the fact that compounds with poor estrogenic potency lack the ability to elicit this second nuclear peak. Injection of an estrogen derivative with slow release properties showed that the two receptor peaks remained temporally constant to each other with translocations occurring at 7 and 16 hrs respectfully. The possibility of enterohepatic pharmacokinetics of the estrogenic compounds contributing to the multiple receptor peaks was investigated by performing these uterine temporal pattern distribution studies in germ-free mice which possess no bacterial flora and, consequently, no enterohepatic recirculation. Experiments with the germ-free mice showed the same multiple temporal pattern as non germ-free mice suggesting that these events were not due to a pharmacokinetic anomaly. Steroid autoradiography techniques were used to demonstrate that the two events are occurring in different uterine cell types. After an IP injection of labeled estradiol there is a temporal pattern of interaction observed with the hormone appearing in the nuclei of stromal and glandular epithelial cells and later in luminal epithelium. Experiments are underway using intraluminal injections of hormone to determine if the poor localization in epithelial cells is due to intracellular mechanisms such as inactive receptor or whether there is low or improper uptake of hormone into these cells. The role of this event in estrogen action in the mouse reproductive tract, with regard particularly to the actions of hormonally active environmental chemicals, is being studied since DES treated animals appear to have an altered pattern of receptor depletion/replenishment. Tissue responses involving receptor synthesis, induction of progesterone receptor, RNA polymerase activities, DNA polymerase activities and DNA synthesis are being investigated. A molecular marker for estrogenic activity in uterine tissue is being sought to determine the activity and mechanism of action of hormonally active chemicals. Protein labeling experiments using [^{35}S] methionine and two dimensional gel electrophoresis have illustrated several proteins (32,000 - 54,000) mw range) in uterine tissue from estrogenized animals. Non-enzymatic separation of the three uterine tissue compartments have indicated that some of these proteins are unique to one cell type. These gels have been computer analyzed for qualitative and quantitative differences to determine estrogen responsive protein domains which may exist in different uterine cell types. Preliminary results show that the epithelial and

stromal tissue compartments exhibit significant differences in protein patterns. Estrogen stimulates high molecular weight proteins in epithelial cells while in the stroma low molecular weight components are most prevalent. Proteins from the epithelial compartment also show significant isoelectric charge trails suggesting glycoprotein structure. A labeled 79,000 mw protein with multiple isoelectric points was also found in the incubation media. Incubations with uteri from control animals did not show the presence of these proteins. Studies are underway using labeled galactose and fucose reagents as well as neuraminidase digestion to determine the glycoprotein nature of these intracellular and secreted estrogen responsive proteins. Certain uterine proteins on the gels are being identified by antibody localization. This is accomplished by electroblotting the proteins from the two dimensional gels onto nitrocellulose sheets for analysis by immunoprecipitation. Attempts will also be made to produce a poly (A^+) mRNA preparation and library to study the mechanism of their synthesis. Two dimensional gel electrophoresis was used to analyze estrogen receptor which was partially purified from mouse uteri by steroid affinity chromatography. Preparations were identified by silver staining as well as blotting on to nitrocellulose paper. Preparations were immunoblotted with ^{125}I estrogen receptor antibody. Fluorographs of these blots produced 2 spots at 70,000 mw at two different isoelectric points (pI 6.8 and 6.5). Studies are planned using differential isolation and ^{32}P labeling procedures to determine if the two receptor specific spots represent the nuclear and cytosolic forms of the receptor and whether the more acidic form may be a phosphorylated form of the estrogen receptor.

The association of in utero DES exposure and reproductive tract cancer in human beings is well documented. Recent development of a mouse model to study these effects will allow this problem to be more fully investigated. The objectives of this project are to define the roles of receptor protein-chemical interactions and the biochemical mechanisms associated with the toxicologic responses observed in the reproductive tract following in utero exposure to hormonally active environmental chemicals.

B. PLANS FOR SUBSEQUENT YEAR

When the estrogen receptor is activated it has an increased affinity for DNA. This property is believed to explain its nuclear localization and interactive nature with the genome. Receptor complexes bound to DES compounds having varying structure will be analyzed by DNA cellulose binding assays. Such information should help determine what chemical structure of the ligand is involved with receptor activation. Different DES receptor complexes will also be tested for their ability to interact with nuclear acceptor protein (NAP) extracted from target tissue chromatin. It would be expected that the two assays will be correlative since both involve analyzing the ability of estrogen receptor to interact with various genomic components. If certain complexes do not bind as well as the DES complex, then it is suspected this may be the reason for their poor biological activity. One particular compound (Indenestrol A) is an in vivo DES metabolite which possesses a chiral center and exists as two enantiomers. The enantiomers will be separated by use of a chiral matrix

column and obtained in pure form. Attempts will be made to deduce the absolute configuration of the enantiomers by either ESR or x-ray diffraction analysis. The estrogen receptor binding activity of the individual enantiomers will be assayed in order to demonstrate if the receptor binding site exhibits chiral recognition. In addition, the in vivo indenestrol metabolite will be isolated and analyzed in order to determine which enantiomer is produced. DES is metabolized in target tissues to Z,Z-dienestrol through a p-quinone intermediate. One possible role of the estrogen receptor in DES toxicity is to bind and localize reactive DES compounds within target tissues. In order to test this idea, binding studies of unlabeled DES p-quinone will be done to determine if there is any binding activity by the receptor. Additionally, receptor reactions will be performed with tritium labeled DES p-quinone in order to directly determine the binding affinity and to analyze whether DES quinone may bind irreversibly to the estrogen receptor.

Earlier studies have indicated that multiple nuclear receptor interactions are required for stimulation of uterine growth. The second period of receptor interaction occurs at 7-8 hrs after the first. Attempts will be made to further characterize these two receptor translocation peaks to determine the quantity of salt resistant high affinity chromatin binding sites. Uterine nuclear matrix isolated at different times after estrogen stimulation will be assayed for differences in nuclear receptor binding sites. These approaches should be informative in determining the genomic site of estrogen receptor interaction and its correlation to uterine stimulation and growth.

In an attempt to establish an estrogen specific genomic marker, studies are planned to analyze protein patterns by two-dimensional gel electrophoresis from different uterine cell types. These analyses will be applied to samples from estradiol and estriol treated animals which should differ in their patterns since estriol stimulation induces all uterine responses except hyperplasia. It should then be possible to detect proteins in the estradiol group which are not present with estriol.

In conjunction with these protein gel studies, the uterine estrogen receptor will be localized in the tissue sample by using immunoblotting with labeled estrogen receptor antibody. The approach should also be applicable to determining whether the estrogen receptor is phosphorylated. Once these receptor forms are detected, preparations can be made from tissue fractions at different points of stimulation to determine the level of the phosphorylated and unphosphorylated states. Mechanistically, EGF and its receptor specifically phosphorylate cellular proteins. In order to correlate uterine EGF actions and estrogen specificity, there will be an attempt made to determine if EGF is involved in phosphorylating the estrogen receptor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70067-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*
Molecular Mechanism of Steroid Hormone in Sex Organ Development.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. T. Teng	Expert	LRDT NIEHS
Others:	J. A. McLachlan	Head, Devel. Endo. and Pharm. Section	LRDT NIEHS

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Developmental Endocrinology and Pharmacology

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously reported the DNA sequence of a 3.3 kb Eco R1 fragment which contains the entire transcription unit plus 5'- and 3'- flanking regions of the rat seminal vesicle IV gene (SVS IV). The 5'- flanking region of this gene contains a perfect inverted repeat at -117 bp from the CAP site. The potential for forming a true cruciform structure in this region can be demonstrated in supercoiled configuration. Therefore, by S1 nuclease digestion of the supercoiled plasmid SV3.3 (containing SVS IV) we have shown that there is an S1 nuclease sensitive site near the putative cruciform structure. We also detected an S1 nuclease-sensitive site in the 3' flanking region of SVS IV about 800 bp from the termination site of the transcription unit. Recently we studied the S1 nuclease-sensitive and DNAase I hypersensitive sites in the chromatin of the SVS IV gene. We found that the region around -100 to -150 bp was susceptible to the S1 nuclease. DNAase I hypersensitive sites of SVS IV genes were present in seminal vesicle cells in which the gene is expressed and were absent in liver cells in which the gene is repressed. SVS IV gene is undermethylated in seminal vesicle and is highly methylated in liver. We have purified a estrogen responsive secretory protein from mouse uterus through HPLC. This protein is highly glycosylated. Two-dimensional gel electrophoresis indicated this estrogen stimulated protein has molecular weight of 70 KD with pI 6.5-6.8. Antibody against 70K protein has been raised in rabbits. We are in the process of establishing radioimmunoassay for this protein. Two times oligo-dt column purified mouse uterine mRNA from both immature and E2-stimulated animals have been prepared. With the aid of 70K antibody and poly A mRNA, we are able to isolate the cDNA clone which carry the genetic information for 70K protein from mouse uterine cDNA library.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

One of the most interesting and poorly understood problems in developmental biology is the expression of sets of hormone regulated genes in a tissue specific way. Using the technology presently available, it is possible to approach such a problem in a relatively detailed way and facilitate a better understanding of differentiation and development of hormone regulated genes.

Objective:

The objective of this project is to study the mechanism involved in steroid hormone regulated gene expression, sex differentiation and development. The goals are: (1) The gene for the seminal vesicle secretion protein IV in rat (rSVS IV) is under detailed study; (2) DNAase I and S1-nuclease sensitive sites in chromatin structure around the rSVS IV gene are presently being defined; (3) The rSVS IV cDNA probe is used to identify and isolate SVS IV gene from mouse genomic library; (4) Isolate and purify uterine specific estrogen responsive 70K secretory protein; (5) Prepare antibody against 70K protein; (6) Identify and isolate gene coding for uterine specific 70K secretory protein.

Experimental Approach and Scientific Justification:

E. Coli containing plasmids with SVS IV inserts were grown and amplified in L-broth containing chloramphenicol. Plasmids were isolated using CsCl-propiduim di-iodide centrifugation. Restriction digestion and agarose or acrylamide electrophoresis were done by standard procedures. Probes of cloned DNA fragments were prepared with ^{32}P-NTP and nick-translation. Southern hybridizations were performed by standard techniques. Detection of supercoiled plasmid and subsequent linearization with S1-nuclease were analyzed using Tris-acetate buffer in agarose gel electrophoresis. Isolation of nuclei was performed using the standard heavy sucrose method. The purified nuclei were digested with either DNAase I or S1-nuclease. The DNA were then extracted and purified with phenol; chloroform method.

Mouse uterine 70K secretory protein was isolated through a series of column chromatography. Rabbit polyclonal antibody against uterine 70K protein were obtained through standard immunization procedure.

Recent Accomplishments and Significance:

The androgen dependent rat seminal vesicle secretion IV gene has been cloned and sequenced previously. The 5'-flanking region of this gene contains a perfect inverted repeat at -117 bp from the CAP site. The potential for forming a true cruciform structure in this region can be demonstrated in supercoiled configuration. Therefore, by S1-nuclease digestion of supercoiled plasmid pSV3.3 (containing SVS IV), we have shown that there is an S1-nuclease-sensitive site near the putative cruciform structure. We also detected a

S1-nuclease-sensitive site in the 3' flanking region of SVS IV about 800 bp from the termination site of the transcription unit. Recently, we studied the S1-nuclease-sensitive and DNAase I-hypersensitive sites in the chromatin of the SVS IV gene. We found that the region around -100 to -150 bp was most sensitive to DNAase I and S1-nuclease digestion. There were several minor nuclease sensitive sites located either 5 kb upstream from the CAP site or 1 kb downstream from the termination site of the transcription unit. The precise locations are currently under investigation. DNAase I-hypersensitive sites and S1-nuclease sensitive sites of SVS IV gene were not detected in chromatin from rat liver in which the gene is repressed. SVS IV gene is undermethylated in seminal vesicle and is highly methylated in liver. The present findings correlated well with the current hypothesis that the presence of DNAase I-hypersensitive sites is necessary for transcription by RNA polymerase II in vivo and the actively expressed gene is generally undermethylated.

The immature mouse accumulated a large quantity of luminal fluid in the uterus (which is enriched with proteins) after receiving E2 for 3 days. Analysis of total proteins from uterine luminal fluid on SDS-PAGE, more than 40 proteins can be resolved. There are two predominant proteins at 70K and 63K dalton region. Partially purified the 70K protein through CM-Affi gel blue column chromatography did not interact with antibody against mouse transferring, albumin and serum which indicated that the 70K protein was a true uterine secretory protein. We have also detected 70K protein in condition medium of uterine epithelium cell culture by SDS-PAGE.

In vitro ^{35}S-methionine labeling experiment shown that the 70K protein is the major protein stimulated by estrogen in both immature and ovariectomized adult female mice. By using two-dimensional gel electrophoresis mapping of the ^{35}S-methionine labeled proteins secreted by unstimulated and E2-stimulated uteri, we found the major protein stimulated by E2 appear at 70K with PI 6.5-6.8. PAS-stain of the SDS-PAGE indicated this 70K protein is a glycoprotein. At present, we have further purified the 70K protein through HPLC (reverse phase) to 95% pure. Rabbit polyclonal antibody against the pure protein have been obtained and total poly A mRNA from both immature and E2 stimulated mouse uteri have also been prepared.

B. PLANS FOR SUBSEQUENT YEAR

We are in the process of making cDNA library from E2-stimulated total poly A mRNA. With the aid of 70K antibody and poly A mRNA from both unstimulated and E2-stimulated uteri, we are able to identify the 70K cDNA clone. Eventually, we will isolate the 70K uterine secretory protein gene and will use as a marker to study hormone regulated sex organ differentiation. Radioimmunoassay of 70K protein will be established and serve as another approach to study hormone action and organ development.

By using the cloned rSVS IV (rat SVS IV) cDNA as a probe we are going to analyze the distribution of SVS IV sequences in the mouse genome. The rSVS IV cDNA probe will then be used to screen by in situ plaque hybridization the mouse genomic library in bacteriophage λ. Similar molecular analysis of the rat SVS IV gene will be carried out in mouse SVS IV gene.

Using the technology presently available, a relatively detailed model of how steroid hormones act on their target tissues can be obtained in the near future. With the androgen regulated SVS IV gene from seminal vesicle and the estrogen regulated 70K gene from uterus of the mouse as molecular markers, we can perhaps better understand the variety of steps involved in gene function, sex differentiation, and development.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70069-02 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Peptide Growth Factors in Reproduction and Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. P. DiAugustine	Research Chemist	LRDT NIEHS
Others:	R. M. Pratt	Head, Exp. Teratogenesis Section	LRDT NIEHS
	R. I. Grove	Staff Fellow	LRDT NIEHS
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	J. A. McLachlan	Head, Devel. Endo. and Pharm. Section	LRDT NIEHS
	K. S. Korach	Research Endocrinologist	LRDT NIEHS
	C. T. Teng	Expert	LRDT NIEHS

COOPERATING UNITS *(if any)*
University of North Carolina (Chapel Hill)
Chiron Corporation

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Developmental Endocrinology and Pharmacology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epidermal growth factor (EGF) is a polypeptide capable of stimulating proliferation of a wide range of cell types in vitro. In an attempt to further purify EGF from various commercial sources, we noticed that multiple forms of the polypeptide could be demonstrated by reversed-phase high-performance liquid chromatography. The two major forms, α- and β-EGF, were analyzed to determine any properties unique to that originally reported for EGF. The physicochemical properties of the two peptides were essentially identical but analysis of the two peptides by mass spectrometry and amino acid sequencing indicated the α-EGF had the primary structure originally reported for EGF, and that β-EGF was missing the amino-terminal asparagine. This [Des-Asn^1]-EGF was significantly less potent than α-EGF in stimulating proliferation of human embryonic palatal mesenchyme cells. In a second study, ^{125}I-α-EGF was injected intravenously to examine the potential of this peptide to cross the placental barrier in CD-1 mice at different stages of pregnancy (10, 13, and 17 days). At the earliest periods that counts could be detected in embryos (5-10 min) after injection of the trace, embryos were removed, extracted, and the extracts examined by gel filtration; only free ^{125}I was detected. Intact ^{125}I-α-EGF could not be found in plasma or other tissues 5 min after injection. In vitro studies indicate that the peptide can be rapidly degraded to amino acids plus monoiodotyrosine (MIT) by the placenta; some tissues, such as the kidney, can dehalogenate MIT to account for the free ^{125}I that crosses the placenta. The intact peptide, however, exhibits no placental transfer. In a third study, we are exploring the potential role of EGF to mediate estrogen-induced proliferation of uterine epithelial cells. Our present data suggest that mouse uterine epithelial cells in vitro are responsive to EGF, exhibit specific binding sites, and reveal positive cytoplasmic immunolocalization of the polypeptide in vivo.

PROJECT DESCRIPTION

A. RESEARCH PROJECT I.

Chemical and Biological Properties of Multiple Forms of Mouse Epidermal Growth Factor

Nature of Problem:

Purification of mouse submaxillary gland epidermal growth factor (EGF) by open column methods was originally thought to provide a homogeneous preparation of this polypeptide. However, subsequent purification of EGF by reversed-phase high performance liquid chromatography revealed at least two forms of EGF.

Objective:

The objective of our investigation was to analyze α- and β-EGF obtained by HPLC and determine specifically any structural or compositional properties different from those previously reported for EGF.

Experimental Approach and Scientific Justification:

The two forms of EGF were separated by an HPLC system consisting of two μBondapak C_{18} columns in series; elution was carried out at 22°C with 25% (v/v) acetonitrile - 10 mM diethylenetriamine phosphate, pH 7.0. Fractions obtained were monitored for both absorbance ($A_{280\,nm}$) and radioreceptor activity with purified rat plasma membranes. ^{125}I-α-EGF was the radioligand for radioreceptor assays.

Recent Accomplishments and Significance:

Amino acid analysis of α- and β-EGF indicated no significant difference in composition. In addition, various other chemical and enzymic methods to convert α- to β-EGF or alter the ratio of the two forms were unsuccessful. Both peptides could be cleaved with CNBr indicating that each peptide did not have an oxidized methionine residue. The first clue as to the difference between these peptides came when the mass spectrum of α-EGF indicated a size equivalent to that originally proposed for EGF, whereas that for β-EGF was significantly lower in mass equivalent to the loss of an aspartic, asparagine, leucine, or isoleucine residue. Since β-EGF elutes later than α-EGF in the HPLC isocratic system and since asparagine is known to offer a negative hydrophobic contribution to peptides, we considered that β-EGF might be a des-asparaginyl form of EGF. Furthermore, we figured that a loss of this residue would have to occur near a terminal end of the molecule since reduction of both peptides with 50 mM dithiothreitol - 6 M guanidine HCl - 0.1 M Tris HCl, pH 7.8, created no fragments or major differences in the HPLC retention time of α- and β-EGF; both peptides, however, did become much more hydrophobic after reduction. Confirmation of β-EGF as a des-asparaginyl form of EGF came when the α- and β-peptides were sequenced by Edman degradation. The first eight cycles revealed

the sequences shown below.

α-EGF: Asn-Ser-Tyr-Pro-Gly-X-Pro-Ser

β-EGF: Ser-Tyr-Pro-Gly-X-Pro-Ser-Ser x = unidentified residue

Thus, the sequence analysis confirms the loss of an asparagine residue and indicates that the loss occurs at the NH_2-terminal region of EGF, making β-EGF a [Des-Asn^1]-EGF.

When the polypeptides were cultured with human embryonic palatal mesenchyme cells, the α-form was significantly (three- to four-fold) more potent than the β-form in stimulating cell proliferation. This suggests that the NH_2-terminal region of EGF is important for the mitogenic activity of this peptide.

B. PLANS FOR SUBSEQUENT YEARS

Although no more structural studies related to EGF are in progress the above findings should enable us to routinely obtain homogeneous, and chemically-defined preparations of α-EGF and β-EGF (Des-Asn^1-EGF) for other current or proposed biological studies.

A. RESEARCH PROJECT II.

Evaluation of Murine Placental Degradation and Transfer of ^{125}I-α-Epidermal Growth Factor (EGF)

Nature of Problem:

It is well known that EGF can stimulate the proliferation of a wide variety of cells of ectodermal and mesodermal origin *in vitro*. Previous studies have also shown that EGF can: (1) exhibit specific binding *in vitro* to several mouse embryonic tissues and also membranes from the placenta and amnion; (2) stimulate down-regulation of embryonic cell EGF receptors; and (3) stimulate incorporation of ^{3}H-thymidine into DNA of several tissues of mouse embryos *in vitro*. A mitogenic effect of EGF has also been shown for a variety of murine embryonic cell lines. The above studies suggest that EGF--or a related peptide--has a role in embryonic/fetal growth. The source of an EGF-like peptide may be either endogenous synthesis by embryonic tissues, including placenta, or placental transfer of maternally-derived blood-borne EGF.

Objective:

In the present study, we examined the placental transfer and degradation of ^{125}I-α-EGF administered intravenously to pregnant mice.

Experimental Approach and Scientific Justification:

^{125}I-α-EGF injected intravenously into 10-, 13-, or 17-day pregnant CD-1 mice is cleared and degraded extremely rapidly (t 1/2 < 1 min). Some plasma (maternal) samples taken 1 min after injection, revealed radioactivity associated with ^{125}I-EGF by gel filtration. However, in all samples taken 5 min or more after injection, radioactivity was only associated with free ^{125}I and material much larger than EGF. Direct degradation by maternal plasma was not evident.

Time-course experiments showed that binding to or incorporation of trace rapidly occurs with placentas but is relatively delayed in conceptuses. Extracts of placentas at different intervals following injection revealed radioactivity shifting from a size near that of EGF to material eluting as ^{125}I. Only free ^{125}I was detected in extracts of conceptuses taken 5-15 min after injection of ^{125}I-α-EGF. Even after coinjection of 5 μg unlabelled α-EGF with trace, intact ^{125}I-EGF could not be detected in conceptuses.

In vitro degradation studies support that the placenta readily degrades ^{125}I-EGF to monoiodotyrosine, but does not dehalogenate this product. However, kidney tissue in vitro degrades ^{125}I-α-EGF to monoiodotyrosine (MIT) plus free iodide and can dehalogenate ^{125}I-MIT to form free ^{125}I. Thus, the ^{125}I found in plasma and extracts of placenta and conceptuses resulted from a dehalogenase present in kidney and probably other tissues. ^{125}I-α-EGF is probably degraded to constituent amino acids and ^{125}I-MIT in lysosomes; the ^{125}I-MIT is dehalogenated by some tissues to free ^{125}I.

Recent Accomplishments and Significance:

Since previous studies have shown that placental transfer of ^{125}I readily occurs, it is reasonable to find this material in conceptuses after ^{125}I appears in plasma following maternal degradation/dehalogenation of ^{125}I-EGF. The delay in incorporation of label in conceptuses is probably related to the lack of placental transfer of intact ^{125}I-α-EGF and the time required to degrade the labelled peptide to free ^{125}I in extraplacental tissue. Maternal extraplacental degradation scheme ^{125}I-α-EGF → ^{125}I-MIT → ^{125}I can account for the presence and uptake of ^{125}I in conceptuses as well as the second phase of uptake (or increased levels) of ^{125}I- radioactivity observed in placentas or plasma.

These findings concur with other studies that found that placental transfer of intact polypeptide growth factors does not occur in mammals.

B. PLANS FOR SUBSEQUENT YEAR

While the above work was in progress, reports from other laboratories have indicated that a peptide is present in mouse embryo extracts that has EGF radioreceptor activity but not EGF immunoreactivity. It was proposed that this peptide may be related to α-transformation growth factor (TGF). TGF has been isolated and purified in another laboratory from the serum-free medium of sarcoma virus transformed rat embryonic fibroblasts. Amino acid sequence of TGF

reveals strong structural similarities with EGF but enough sequence disparity to account for low or negligible cross-immunoreactivity with EGF.

The very low levels of apparent TGF in mouse embryos led us to design a radioimmunoassay for direct determination of this peptide. To accomplish this, the 1-15 amino-terminal region of TGF, or Val-Val-Ser-His-Phe-Asn-Lys-Cys-Pro-Asp-Ser-His-Thr-Gln-Tyr-amide, was custom synthesized and coupled through the free -SH to Concanavalin A with the heterobifunctional reagent N-succinimidyl 3-(2-pyridyldithio) propionate and used as an antigen. The development of a successful RIA should, then, provide a means for determining relationship of embryonic α-TGF levels to development.

A. RESEARCH PROJECT III.

Investigation of a Putative Role of Epidermal Growth Factor in Mediating Estrogen-Induced Proliferation of Uterine Epithelial Cells.

Nature of Problem:

It is well known that 17β-estradiol (E_2) and synthetic estrogens can stimulate *in vivo* proliferation of epithelial cells of the uterus, mammary gland, and vagina. In collaboration with several investigators, various experimental approaches have been designed or are currently in progress to explore the notion that the mitogenic action of estrogens is mediated through a growth factor such as EGF. Testosterone-induced stimulation of EGF levels in male and female submaxillary glands is well documented. Perhaps an estrogen-induced stimulation of EGF can occur in female reproductive tract tissues and accessory organs.

Objective:

The studies in progress described below represent an effort to examine mouse uterine tissue for potential *de novo* synthesis and mitogenic action of EGF and the relationship of estradiol to uterine EGF levels. These studies constitute most of my current research activities.

Experimental Approach and Scientific Justification:

Immunohistochemistry. Using the antibody-bridge technique and applying high affinity rabbit antiserum to mEGF sections of uteri from female mice treated with estrogen, we observed distinct cytoplasmic staining of mouse uterine epithelial cells, both glandular and luminal. Staining could be markedly reduced by adding mouse EGF to the reaction mixture. We plan to contrast estrogen treated and control adult ovariectomized and immature mice in regard to staining intensity.

Staining in the uterus, so far, was much less than that produced by ductal cells of the mouse submaxillary gland. We intend to extend these studies to include the mouse mammary gland. Electron microscopy of adult mouse uterine

epithelial cells and stromal cells reveal no cell populations containing granules typical of those observed in the ductal EGF-containing cells of the submaxillary gland.

Radioimmunoassay. Studies are in progress to measure EGF by radioimmunoassay in whole uterine extracts and in uterine luminal fluid. Chromatographic (HPLC and gel filtration) procedures will be performed to assess the similarity of physicochemical properties of the immunoreactive peptides with those of EGF. These studies will include comparisons of E_2-treated and saline-treated adult ovariectomized mice, as well as investigation of the time-course of any elevations of EGF-like immunoreactivity caused by estrogen treatment. We also propose to challenge any elevations in vivo caused by E_2-treatment with inhibitions of DNA-dependent RNA synthesis (actinomycin) and protein synthesis (puromycin).

Cell Culture Studies. We have evidence that clearly shows specific, saturable, high-affinity binding of ^{125}I-α-EGF to primary cultures of mouse uterine epithelial cells prepared by Dr. Y. Tomooka. This finding complements his studies that show that EGF in a dose-dependent manner stimulates proliferation of these cells in vitro. We are extending these studies to investigate the potential for estradiol to stimulate EGF levels in uterine epithelial cells in vitro. Both cells and conditioned medium will be examined for EGF by radioimmunoassay. We also propose to investigate the in vitro growth-inhibitory effect of EGF antisera on poliferating uterine cells that have been deprived of exogenous EGF.

Passive Immunization Against Estradiol-Induced Uterine Cell Proliferation. Preliminary data in our laboratory suggest that pretreatment of immature (17 day old) female mice with antisera to EGF may inhibit diethylstilbestrol-induced increase of uterine weight. An experiment has been designed to compare the effects of EGF-antibody, nerve growth factor (NGF) antibody, rabbit gamma-globulins in altering the extent of DES-induced uterine cell proliferation. We plan to examine changes in wet weight, dry weight, total DNA [^{3}H]-thymidine incorporation, and histological appearance.

Dot Blot Hybridization. We obtained a fragment of the cDNA for mouse prepro EGF. This fragment, pmegf 10, which overlaps the region coding for EGF sequence will be used for hybridization experiments on nitrocellulose paper. With this method we propose to examine mouse uterine and other tissues for stimulation of mRNA EGF following estrogen treatment. Hybridization experiments with the ^{32}P-pmegf 10 probe, so far, clearly indicates, as expected, much higher levels of EGF RNA in the male mouse submaxillary gland than in estrogen-treated immature female mice. Our preliminary data indicate that elevation of uterine mRNA-EGF may occur in those animals treated with estrogen. The availability of this probe provides us with a means to directly determine any increment of uterine mRNA EGF as a genomic response of estrogen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70075-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*
Nature and Role of Germ Cell Surface Molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. M. Eddy	Head, Gamete Biology Section	LRDT	NIEHS
Others:	D. A. O'Brien	Senior Staff Fellow	LRDT	NIEHS
	I. P. Lee	Pharmacologist	LRDT	NIEHS
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Fred Hutchinson Cancer Research Center, Seattle
University of Washington School of Medicine
The Hospital for Sick Children, Toronto, Canada

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Laboratory of Reproductive and Developmental Toxicology

SECTION
Gamete Biology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gamete cell surface components confer specificity in events critical for processes of reproduction and development. Antibodies can block gamete function and embryogenesis, but the specific molecules involved have not been identified. Monoclonal antibodies are being produced to characterize these molecules, determine their origin and distribution, and define their roles. There are three aspects of the project. (a) One study has examined the appearance of new sperm surface components during epididymal maturation, coincident with sperm gaining the ability to swim and to fertilize. A 54,000 molecular weight antigen being studied is secreted by epithelial cells in the caput epididymidis and attaches specifically to acceptor sites on the plasma membrane of the flagellum. It is cell type and species specific, present in the epithelium after two weeks of age, absent from sperm recovered from the oviduct, lost during in vitro capacitation, but retained on sperm incubated in the presence of mouse or rat epididymal fluid. (b) An additional study has examined sperm surface components that may be involved in the acrosome reaction. Surface molecules are lost during the acrosome reaction as shown by using a monoclonal antibody to monitor antigen loss and a protease activity assay to measure calcium-stimulated acrosomal enzyme release. The protease release was inhibited in sperm exposed to antibody prior to calcium treatment. (c) Another study has examined surface antigens shared by germ cells, teratocarcinoma cells, and embryonic cells. Some of these are high molecular weight glycoproteins and stage-specific expression of particular antigens during spermatogenesis is due to changes in patterns of sialic acid incorporation during post-translational modification of the glycoconjugate.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Gametes bear surface components that confer specificity in cell recognition, attachment, and other cell surface events, but the specific molecules have not been identified. Antibodies to gametes, teratocarcinoma cells, and embryonic cells cross-react and also block events of reproduction and development *in vivo* and *in vitro*.

Objectives:

The objectives of this project are to characterize these molecules, determine their origin and distribution, and define their roles in processes of reproduction and development.

Experimental Approach and Scientific Justification:

The approach being used is to produce monoclonal antibodies against cell surface components or isolated molecules and to use those highly specific probes in morphological, biochemical, and physiological studies on germ cells of rodents.

The nature of sperm surface components and their time and site of appearance must be known to understand their roles in reproduction. Once individual surface components are identified as to composition, topographic distribution and origin, their possible participation in the events of gamete formation, sperm maturation in the epididymis, capacitation in the female reproductive tract and in fertilization can be studied systematically. These studies provide information of value in the design of strategies for regulation of male fertility, in the diagnosis and treatment of male infertility, and in understanding the effects of environmental agents on male reproductive processes.

Recent Accomplishments:

(1) Characterization of sperm surface components appearing in the epididymis. Hybridoma cell lines were produced by fusing myeloma cells with spleen cells from male mice immunized with syngeneic sperm from caudae epididymides and vasa defferentia. Clonally derived cell lines were selected which secreted monoclonal antibodies against sperm surface components. Each of the antibodies bound to a specific region of the sperm surface as seen by indirect immunofluorescence (IIF) microscopy. They recognized topographically restricted antigenic determinants either on different regions of the head or restricted to the tail of mouse sperm. Some of the monoclonal antibodies bound to sperm from other species of mammals and the patterns of antigen distribution differed between species.

When sperm from different regions of the reproductive tract of male mice were examined, the antigens were not detected on most sperm until they had reached

the distal caput or corpus epididymidis. The appearance of new sperm surface antigens during epididymal maturation may be due to the insertion of components from the sperm cytoplasm, the exposure or modification of pre-existing surface components or the attachment of new components to the sperm surface. Four individual antigens were examined to determine their origins, using immunohistochemistry on cryotome sections of unfixed tissue and on paraffin sections of fixed tissue from different regions of the male reproductive tract. One of the antigens was not detected, two were in epithelial cells throughout the caput and corpus epididymidis, and one was found in epithelial cells in a short length of the distal caput epididymidis. This latter antigen, referred to as sperm maturation antigen (SMA) 4 was chosen for further study because of its discrete localization in the epididymis and its subsequent presence on the surface of the sperm tail.

Using the monoclonal antibody, it was demonstrated that sperm retained in the ductuli efferentes following ligation do not acquire SMA 4, suggesting that its appearance is not due to changes intrinsic to the sperm, but that the epididymal environment is required. Because the antigen is present in the epididymal epithelium, ligation experiments were carried out to show that absence of sperm or testicular fluid from the epididymis did not affect production of the antigen. Examination of prepuberal mice indicated that antigen production is age dependent, production beginning in the epididymis of mice between two and four weeks of age. IIF analysis of sections of a variety of tissues and organs determined that the antigen is restricted to sperm and to epithelial cells of the male reproductive tract and is not present in other tissues. In addition, experiments comparing the antibody-induced agglutinability of sperm from the caput epididymidis to that of sperm from the cauda epididymidis confirmed that the antigen resides on the sperm surface. Taken together, these findings indicate that the antigen is secreted from a discrete zone in the epididymal epithelium and attaches to a restricted domain on the sperm surface.

To examine the nature of the association of SMA 4 with the plasma membrane, sperm were exposed to high salt, low salt, high pH or low pH. None of these treatments caused loss of the antigen, as assayed by IIF, indicating that the antigen is attached tightly to the cell surface. The antigen is removed by treatment with low concentrations of nonionic detergent and can be detected in the supernatant by an enzyme linked immunosorbant assay (ELISA). Saccharidases failed to alter the antigen, but trypsin and papain were effective at destroying reactivity, suggesting that the determinant contains protein. It was not removed by EDTA or mercaptoethanol, indicating that it is probably not linked via divalent cation-dependent or disulfide-bond containing moieties. In addition, it was found that the antigen was still present on sperm which had resided in the uterus from 3 to 12 hours, but sperm associated with eggs or cumulus masses flushed from oviducts 5 1/2 hours after insemination lacked the ability to bind the antibody, indicating that the antigen is lost, modified, or masked in the upper part of the female tract. Under in vitro capacitation conditions, approximately 90% of the sperm lost reactivity to the antibody by 3 hours. The loss was not inhibited by lack of energy sources or blockage of oxidative phosphorylation, but it was inhibited by low temperature, lack of protein in the medium or presence of mouse or rat epididymal fluid. It is of

interest that SMA 4 is not present in the rat, but rat epididymal fluid contains the stabilizing factor. Enzyme inhibitors failed to block loss of the antigen.

Biochemical characterization of SMA 4 indicated that the antigenic determinant is carried on a 54,000 MW component, as determined by PAGE and immunoblotting procedures. The molecule does not stain with Coomassie blue, but is PAS positive, suggesting that it is a highly glycosylated protein.

(2) The role of sperm surface components in reproductive processes. Bioassays are being developed to examine the roles of gamete cell surface components. Those in use now include in vitro model systems of capacitation, the acrosome reaction, and fertilization. Monoclonal antibodies are being used to monitor cell surface events during these processes or to determine the effects of antibodies on them. The loss or change in distribution of individual molecular species can be followed, and antibodies which block or perturb such processes identify molecules with putative roles in these processes.

The acrosome reaction involves a fusion between the plasma membrane and the outer membrane of the acrosome and the subsequent release of acrosomal enzymes required for the sperm to penetrate the egg's investements. Sperm surface components may be involved in the acrosome reaction, especially those components restricted to the marginal zone (anterior acrosome) of the acrosome when the process begins. Molecules on the equatorial segment (posterior acrosome) or on the plasma membrane over the entire acrosome could be important in the acrosome reaction as well. IIF studies demonstrated that a sperm surface component over the anterior acrosome, detected by a monoclonal antibody, was lost with the acrosome reaction. Using the sensitive BAEE assay for sperm protease release as an indicator of the Ca^{++}-stimulated acrosome reaction, it has been found that protease release was inhibited in sperm exposed to that monoclonal antibody prior to Ca^{++} treatment. These findings indicate that monoclonal antibodies can be used to monitor changes in sperm surface components during the acrosome reaction and that they may permit the identification of individual sperm surface components that participate in particular aspects of the event. Other monoclonal antibodies and lectins which bind to the sperm surface over the acrosome are being assayed to determine their effects on this process. It is predicted that some will have no effect and others will accelerate the acrosome reaction by perturbing the participation of different components in the membrane motility processes involved in this exocytosis event.

To examine the role of individual sperm surface components in the fertilization process, mouse sperm are being treated with monoclonal antibodies which bind to different regions of the surface and used in in vitro fertilization assays with mouse eggs. Superovulated eggs with intact cumulus cell masses were examined 6-8 hours after insemination and were considered fertilized if two of three criteria were met: presence of two pronuclei, presence of sperm tail in the cytoplasm, and presence of a second polar body with condensed chromatin. Syngeneic antiserum to mouse sperm reduced fertilization to below 10% and was used as a positive control. Of the 34 monoclonal antibodies tested thus far, 7 reduced fertilization to 10% or less, while the others had partial or no effects. Additional antibodies remain to be assayed and further studies will

be necessary to determine if fertilization is inhibited at the level of the cumulus cells, the zona pellucida, or the egg surface, and to identify the sperm surface molecules involved.

(3) Analysis of surface antigens shared by germ cells, teratocarcinoma cells, and embryonic cells. Monoclonal antibodies have been produced which recognize surface components of germ cells, teratocarcinoma cells and embryonic cells, confirming the observations of others that these cell types have common surface antigens. This indicates that common genes are active in these cell types and suggests that the gene products are markers of pleuripotent cells or that the cells share common functional activities.

Three of these antigens have been partially characterized and have been shown to be glycoproteins, with the determinants recognized by the monoclonal antibodies (C6, A5, and J1) carried on glycoconjugates. C6 and A5 identify cell surface carbohydrates shared by different teratocarcinoma cell lines and early mouse embryos. The binding of both to F9 teratocarcinoma cells was inhibited by N-acetyllactosamine. A5 agglutinated adult, but not fetal, erythrocytes of both type A and O, suggesting partial specificity for branched polylactosamine structures. C6 and A5 did not label preimplantation stage embryos; however, both antibodies labeled embryos following neuraminidase treatment. In paraffin sections of postimplantation stage embryos, both antibodies exhibited similar patterns of labeling, being localized mainly at the luminal surfaces of ectodermal and visceral endodermal epithelia. Neuraminidase treatment exposed additional binding sites in the ectoderm and mesoderm of postimplantation embryos, not restricted to periluminal surfaces. These results suggest that cell surface carbohydrates are modified during early embryogenesis by sialylation.

J1 does not react with embryos but, along with C6 and A5, does recognize antigens present on teratocarcinoma cells and mouse germ cells but not on the adult somatic tissues tested. They label the surface of living, isolated early and late pachytene spermatocytes and round spermatids. Germ cells from fetal and neonatal testes were not labeled, but in paraffin sections of prepuberal and adult testes, sialidase treatment exposed antigens recognized by C6 and A5 on earlier stage germ cells. The binding of J1 to adult testicular cells was inhibited by N-acetylglucosamine and the binding of both C6 and A5 was inhibited by N-acetyllactosamine. The glycoconjugates recognized by J1, C6, and A5 eluted from gel filtration columns with an apparent molecular weight greater than one million and were sensitive to endo-β-galactosidase treatment. The results suggest that the molecules which carry these determinants on meiotic and haploid germ cells undergo changes in sialylation during the transition of early meiotic germ cells to pachytene spermatocytes in spermatogenesis.

Primordial germ cells (PGC) have been hypothesized to originate from pleuripotent stem cells in the primary ectoderm or to arise in the primitive endoderm under epigenetic influences. Two monoclonal antibodies (EMA-1 and EMA-6) have been used to immunostain sections, identify PGC in mouse embryos between days 8 and 13 of development, and test these hypotheses. In day 8 embryos, EMA-1 identified PGC in the mesoderm beneath the caudal end of the primitive streak, at the base of the allantois, and in the endoderm of the forming hindgut.

The entire cell surface and a cytoplasmic granule were stained. On day 8.5, PGC sat adjacent to the endoderm of the hindgut invagination. At 9 days, EMA-1 and EMA-6 labeled PGC within the hindgut endoderm or entering the dorsal mesentary and by 10 days, most PGC were in the dorsal mesentary or entering the urogenital ridges. EMA-1 reacted with PGC in sexually undifferentiated genital ridges at 11 days and in male gonads of 13 day embryos, but not with germ cells at later stages. The antibodies also bound to 8-cell embryos, outer cells of morulae, trophectoderm of expanding blastocysts, primary ectoderm of egg cylinders and embryonic ectoderm of postgastrulation embryos. Although determinants recognized were not exclusive to PGC, the distribution is consistent with the hypothesis that PGC arise from pleuripotent precursors in the primary ectoderm. To test the alternate hypothesis, primitive endoderm was removed from 7.5 day egg cylinders by immunosurgery and the embryos were cultured for 40 hours, through the gastrulation period. PGC were present at the end of this period, indicating that an intact primitive endoderm is not required for their appearance in the embryo.

Preliminary results indicate that the antigenic determinants recognized by EMA-1 and EMA-6 on Nulli teratocarcinoma cells reside on richly glycosylated high molecular weight glycoproteins. Both antibodies reacted with some, but not all, mouse and human teratocarcinoma cell lines tested. EMA-1 reacted with mouse brain, kidney, and sperm, but not testes; EMA-6 reacted with kidney, but not brain, sperm, or testicular cells. A syngeneic antiserum to Nulli teratocarcinoma cells gave a similar combined pattern of tissue reactivity. The results of these studies and those of other investigators indicate that teratocarcinoma cell lines differ in the expression of surface antigenic determinants shared with germ cells and embryonic cells. However, the studies with monoclonal antibodies C6, A5, and J1 indicated that major changes in antigenic reactivity can occur with small changes in glycosylation. Such post-translational modifications of proteins may be functionally important, but they may also be exaggerating apparent differences between types and stages of cells. Future studies will determine the cell type and developmental distribution of the protein backbone of molecules which carry particular antigenic determinants to determine whether or not the patterns seen with monoclonal antibodies accurately reflect the expression of particular genes.

Significance:

These studies have demonstrated the feasibility of producing monoclonal antibodies against sperm and gametogenic cells by syngeneic immunization and allow one to predict that this approach will provide highly specific and quantitative reagents usable in the study of inadequately understood phenomena involved in reproduction.

These studies have proven that individual antigenic determinants are restricted to specific areas of the sperm surface and that monoclonal antibodies can be used to characterize sperm surface components.

These studies have confirmed that epididymal maturation results in alteration in the sperm surface and have demonstrated for the first time that sperm surface components produced by the epididymal epithelial cells are applied to

topographically restricted areas of the sperm surface. They have also demonstrated that certain sperm surface components are dependent on the androgen environment.

These studies have confirmed that sperm share antigenic determinants with teratocarcinoma cells and have shown that monoclonal antibodies to teratocarcinoma cells recognize topographically restricted determinants on the sperm surface.

These studies have shown that a variety of cell surface antigens appear at different stages in embryogenesis and that similar antigens are present on teratocarcinoma and germ cells, including primordial germ cells.

B. PLANS FOR SUBSEQUENT RESEARCH

Characterization of sperm surface components appearing in the epididymis.

1. The mechanism of attachment of SMA 4 to the surface of the sperm tail will be analyzed to determine if it binds like a lectin or is linked by metabolic processes.
2. The nature and site of production of the sperm membrane acceptor molecule for SMA 4 will be determined to learn if the molecule is unique to sperm and is synthesized in a stage-specific manner.
3. The component in epididymal fluid which stabilizes the binding of SMA 4 to the plasma membrane will be isolated for biochemical and functional study.
4. Other unique sperm surface components appearing in the epididymis will be characterized biochemically and their roles in reproduction tested in functional assay.

Role of sperm surface components in reproductive processes.

1. The effects of capacitation on the presence and distribution of sperm surface components will be tested to identify molecular changes occurring during the preparation for fertilization.
2. The effects on the acrosome reaction of monoclonal antibodies and lectins which bind to the acrosomal portion of the sperm plasma membrane will be examined to identify molecules involved in this process.
3. Additional studies will determine if monoclonal antibodies which block fertilization act at the level of the cumulus cells, the zona pellucida, or the egg surface, as a way to identify the putative sperm surface molecules involved.

Analysis of surface antigens common to germ cells, teratocarcinoma cells, and embryonic cells.

1. Molecules carrying antigenic determinants common to germ cells, teratocarcinoma cells and embryonic cells will be isolated and compared to learn if the same molecules are present on these different cell types.
2. Monoclonal antibodies will be produced to the peptide portion of these molecules, using the in vitro stimulation procedure.

3. The cell type and developmental stage distribution of the peptide backbone portion of the antigens will be examined to determine the expression of developmentally regulated genes for cell surface components.

PUBLICATIONS

No publications to date have been funded by NIEHS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70078-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*
Characterization of Stage-Specific Surface Antigens During Mouse Spermatogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. A. O'Brien	Senior Staff Fellow	LRDT	NIEHS
Others:	E. M. Eddy	Head, Gamete Biology Section	LRDT	NIEHS

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Gamete Biology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

During spermatogenesis, regional specialization of the plasma membrane occurs resulting in a mature gamete with distinctly polarized functional and biochemical properties. One feature of this differentiative process is the appearance of novel surface constituents in a precise temporal sequence. Both immunological and biochemical techniques have been used to characterize germ cell-specific constituents and monitor membrane assembly. Central to these studies are methods for the purification of germ cells at defined stages of spermatogenesis by enzymatic dissociation of adult or prepuberal testes followed by unit gravity sedimentation. Three areas of research have been pursued: (a) Polyclonal and monoclonal antibodies have been used to characterize macromolecules first appearing on the surface of pachytene spermatocytes, coincident with a period of maximal protein synthesis. These constituents are not shared by most somatic cells and include at least ten proteins, a probable lipid constituent, and large polylactosamine glycoconjugates. Some of these components are retained on sperm, restricted to distinct domains on the cell surface. (b) Conditions for the short term culture of spermatogenic cells are being tested. Earlier meiotic stages from 17-day-old animals exhibit more stringent requirements for in vitro maintenance than later stages from adult mice. Media supplementation with growth factors and hormones has resulted in increased viability of the prepuberal germ cells and should facilitate metabolic studies and the development of in vitro functional assays. (c) Protein synthesis in isolated spermatogenic cells has been examined by 2D PAGE and autoradiography following short term culture with [^{35}S]methionine. Synthetic profiles become more complex throughout meiosis. A number of proteins previously identified as surface antigens are synthesized in a stage-specific manner. Germ cell surface constituents exhibiting both tissue and stage specificity are candidates for further studies exploring cell-cell interactions during spermatogenesis and fertilization.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of the Problem:

The surface of the mature spermatozoan has distinct biochemical and functional domains. Molecular mechanisms underlying the establishment of these domains during germ cell differentiation have not been well defined. It is clear, however, from a growing number of biochemical and immunological studies that novel surface constituents are inserted into the plasma membrane in a precise temporal sequence during spermatogenesis. The goals of this project are to characterize germ cell surface constituents displaying tissue and stage specificity, determine patterns of synthesis and metabolic processing of these molecules, and define their functional roles both in mediating cellular interactions within the seminiferous epithelium and in events leading to fertilization.

Objectives:

(1) Use both polyclonal and monoclonal antibodies to identify and characterize immunochemically stage-specific surface antigens on mouse spermatogenic cells.

(2) Define conditions for the short-term culture of isolated spermatogenic cells.

(3) Examine proteins synthesized by isolated spermatogenic cells using two-dimensional polyacrylamide gel electrophoresis and autoradiography.

Experimental Approach, Scientific Justification and Recent Accomplishments:

(1) Immunochemical characterization of stage-specific surface antigens during spermatogenesis. (a) Polyclonal antibodies. Spermatogenic cell antigens appearing on the cell surface during the pachytene stage of meiosis have been identified immunochemically using polyclonal antisera prepared against mouse germ cells at defined stages of spermatogenesis. Four antisera prepared previously against purified populations of spermatogenic cells from the adult testes and excurrent ducts were used to stain Western blots from one- and two-dimensional SDS gels of germ cell plasma membranes. Three antibodies (AP, ARS, ASC) recognize a similar subset of high molecular weight determinants, plus a number of minor constituents which vary qualitatively and quantitatively between antisera. AVDS, prepared against vas deferens sperm, exhibits the least complex binding pattern. These results indicate that at least ten surface constituents appear during the pachytene stage of meiosis, a period where maximal RNA and protein synthesis has been demonstrated. Furthermore, two-dimensional immunoblot comparisons of plasma membranes isolated from late pachytene spermatocytes and round spermatids reveal differences between surface determinants detectable at these two spermatogenic stages.

Antisera prepared against spermatogenic cell populations isolated from prepuberal mice have also been utilized to examine transitions in surface

constituents during the early stages of meiosis. Immunofluorescent binding studies indicate that ALZ, raised against a population of leptotene and zygotene spermatocytes, binds to the surface of early pachytene spermatocytes and germ cells at subsequent stages of differentiation. Stage-specific antigens recognized by ALZ, including both protein and probable lipid, have been localized immunochemically on Western blots from SDS gels. A dithiothreitol-sensitive constituent (M_r ~39,000) has been identified as the major protein determinant present in early meiotic cells but absent in spermatogonia from 8-day-old animals. This antigen is present in preleptotene, leptotene/zygotene, and early pachytene spermatocyte populations. This constituent could remain within the cell during the early meiotic stages and then be incorporated into the plasma membrane during pachynema, thus accounting for the stage-specific surface labeling observed with ALZ. These studies have identified a number of surface constituents that are not shared by somatic cells and are expressed during restricted periods of spermatogenesis. Such stage-specific macromolecules are candidates for further study using monoclonal antibodies to address functional questions concerning cell-cell interactions during germ cell differentiation.

(b) Monoclonal antibodies. Further analysis of stage-specific expression of cell surface antigens was conducted with three monoclonal antibodies (J1, C6, A5) prepared and characterized by Drs. Bruce Fenderson and E. M. Eddy (see Intramural Project Report #Z01 ES 70075-01 LRDT). Carbohydrate antigens recognized by these antibodies are first detected by indirect immunofluorescence on pachytene spermatocytes isolated from 17-day-old mice, the same stage where ALZ surface reactivity appears. C6 and A5 antigens are also detected on earlier meiotic stages following neuraminidase treatment, suggesting that changes in carbohydrate moieties may be an important feature of germ cell surface differentiation.

(2) Short-term cultures of prepuberal spermatogenic cells. Conditions for the short-term culture of spermatogenic cells from adult and prepuberal mice have been examined. Pachytene spermatocytes and round spermatids from adults can be cultured with > 90% viability for at least 24 h in Eagle's MEM supplemented with 10% fetal bovine serum (FBS), 6 mM lactate, 1 mM pyruvate. In contrast, cell suspensions from 17-day-old (17d) testes, containing meiotic germ cells and Sertoli cells, decline to 60-70% viability when cultured under identical conditions. Viability of 17d germ cells does not improve in DMEM, F12, DMEM/F12, RPMI-1640, 199, or STIM-9. The use of FBS decreases viability of the 17d germ cells and has been subsequently omitted. Improvements in viability are obtained by supplementing with growth factors and hormones. TK, an enriched MEM medium without serum developed by Tres and Kierszenbaum for rat testicular cultures, results in viabilities near 80% for 17d cells cultured for 16-20 h. Constituents of this medium include insulin, transferrin, glutamine, sodium pyruvate, retinol, growth hormone, epidermal growth factor, and testosterone. Age differences were noted, with the viability of germ cells from 20d mice resembling those from adults, remaining in the 85-90% range for up to 40 h in MEM, CEM or TK. Like 17d germ cells, those from 18d or 19d mice have higher viabilities in TK than in MEM, suggesting that hormone and growth factor requirements may change markedly during this interval.

(3) Protein synthesis by isolated spermatogenic cells. Spermatogenic cells isolated from 17d and adult mice have been cultured for short periods in MEM containing [^{35}S]methionine. On a dpm/cell basis, mixed cell suspensions from 17 day testes (meiotic germ cells and Sertoli cells) incorporate more than twice as much material into TCA-precipitable material as adult cells (late pachytene spermatocytes, spermatids, and residual bodies) over an 8 h incubation period. Autoradiograms of two-dimensional gels demonstrate incorporation of [^{35}S]methionine into > 300 proteins (M_r 20,000-150,000, pI 4.5-7.5). The majority of these proteins are detected on autoradiograms and silver stained gels of both cell populations, although quantitative and qualitative differences occur. Five purified germ cell populations (preleptotene, leptotene/zygotene, 17d pachytene and adult pachytene spermatocytes, and round spermatids) were also cultured for 4 h with [^{35}S]methionine prior to 2D gel electrophoresis and autoradiography. These results indicate that germ cells synthesize an increasingly complex array of proteins throughout meiosis, with maximal protein synthesis at the pachytene stage.

Significance:

Mammalian spermatogenesis involves the transformation of diploid spermatogonia into highly specialized haploid spermatozoa. Features of this developmental process include continuous remodeling of the plasma membrane concomitant with marked changes in cell size and shape, the temporal appearance of a number of germ cell-specific surface antigens, and regional specialization of the plasma membrane resulting in a mature gamete with distinctly polarized functional and biochemical surface properties. In addition, spermatogenic cells and surrounding Sertoli cells form specialized intercellular junctions which may facilitate the orderly translocation of germ cells within the seminiferous epithelium as differentiation proceeds. Thus, the potential for participation of the plasma membrane in the regulation of spermatogenesis is immense. The availability of purified spermatogenic cell populations, coupled with advanced biochemical and immunological techniques, should facilitate direct analysis of the molecular assembly of plasma membranes in this system and may reveal general principles that are common to other systems of cellular differentiation. Furthermore, the availability of a variety of mutant mouse strains, defective at distinct stages throughout spermatogenesis, should be helpful in determining the function of stage-specific cellular constituents.

A more detailed molecular framework of normal spermatozoan differentiation should provide new insights both for monitoring and modulating male reproductive capacity. New techniques, such as short-term culture methods, may be useful in assessing the effects of toxic agents on germ cells. In addition, the identification and characterization of plasma membrane components unique to spermatogenic cells may facilitate the development of new contraceptive approaches.

B. PLANS FOR SUBSEQUENT RESEARCH

Immunochemical characterization of stage-specific surface antigens. Additional monoclonal antibodies are currently being prepared against plasma membrane fractions from germ cells at defined stages of spermatogenesis. Antibodies

will be screened by ELISA and indirect immunofluorescence on live cells to monitor surface binding on both spermatozoa and earlier spermatogenic cells isolated from adult and prepuberal mice. Topographical localization on spermatozoa and/or stage-specificity will be used as criteria for screening the antibodies with appropriate functional assays (such as the ability of an antibody to modify the acrosome reaction, in vitro fertilization, or interactions between germ cells and Sertoli cells). Antigens of interest will be further characterized by standard immunological and biochemical techniques, including one- and two-dimensional electrophoresis, immunoblotting techniques, enzyme sensitivities, lectin and sugar hapten inhibition assays for glycosylated antigens.

Short-term cultures of prepuberal spermatogenic cells. Additional factors such as FSH, selenium, and other trace elements are being tested to see if further improvements in viability can be attained for prepuberal germ cells. Other criteria for cell function such as ultrastructure and protein synthesis will be assayed, comparing MEM and media supplemented with hormones and growth factors. Monoclonal antibodies J1, C6, and A5, which recognize trypsin-sensitive glycoconjugates, will be used to monitor the reappearance of surface antigens on cultured spermatogenic cells following cell isolation with trypsin. The use of improved media such as TK for longer culture periods should facilitate studies of the synthesis of germ cell surface antigens and the regulation of their production in a stage-specific manner. Furthermore, these studies should facilitate the development of an in vitro assay for monitoring surface interactions between germ cells and Sertoli cells.

Protein synthesis by isolated spermatogenic cells. Studies examining protein synthesis in purified germ cell populations will be completed, focusing on previously described surface molecules exhibiting stage-specificity. In addition, radiolabeled sugars will be used to assess glycosylation patterns of plasma membrane constituents during spermatogenesis. Plasma membrane isolation techniques used for adult spermatogenic cells are currently being modified to permit purification of membrane fractions from prepuberal germ cells. Using these methodologies, the synthetic profiles for individual surface constituents throughout spermatogenesis can be determined, as a way of monitoring stage-specific metabolic processes and their perturbations by experimental treatment.

PUBLICATIONS

No publications to date have been funded by NIEHS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70080-11 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Study of Toxic Effects of Environmental Chemicals on Spermatogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: I. P. Lee Pharmacologist LRDT NIEHS

Others: None

COOPERATING UNITS *(if any)*
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Laboratory of Developmental Pharmacology, NICHHD

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Gamete Biology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
These studies seek to assess mechanisms by which environmental chemicals modify gene expression of male reproductive organs. The following studies are ongoing: (1) the mechanism by which DNA modifiers increase the TCDD-induced P_1-450 mRNA of prostate glands, and (2) the study of possible mechanisms for potentiation of TCDD-activated prostatic P_1-450 gene by DNA modifying agents. This project is being incorporated into project number Z01 ES 70085-07 LRDT.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70085-07 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Developoment of In Vitro Models For Assessing Reproductive Toxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	I. P. Lee	Pharmacologist	LRDT	NIEHS
Others:	D. W. Nebert	Chief	DP	NICHHD
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COOPERATING UNITS *(if any)*
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Laboratory of Developmental Pharmacology, NICHHD

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SECTION
Gamete Biology Section

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NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies seek to assess the effects of environmental agents on spermatogenesis, sperm maturation, functions of accessory organs, and male reproductive capacity. Mechanisms of toxicity are studied, and new approaches to toxicity testing are proposed and validated in order to extrapolate more reliably from laboratory animals to man and to improve our ability to analyze risk. The following studies are in process: (1) to define the biochemical nature of the molecules of epididymal monoclonal antibodies, the site of action, and the origin of sperm surface glycoproteins; (2) the study of the functional role of the sperm surface molecules and the identification of environmental chemicals which perturb these molecular processes; and (3) mechanisms by which environmental chemicals modify gene expressions of male reproductive organs.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of the Problem:

Reproductive toxicity induced by drugs, industrial chemicals, and other environmental chemicals has become a serious public concern. Consequently, a variety of both in vitro and in vivo toxicity test systems have been developed to help determine male reproductive toxicity. Presently, most reproductive test methods remain descriptive and superficial. Mechanisms of toxicity induced by reproductive toxins are not clearly understood because molecular events of spermatogenic cell differentiation and epididymal sperm maturation remain largely unknown. These studies seek to elucidate the possible mechanisms of epididymal sperm maturation and determine the effects of environmental chemicals on sperm maturation.

Objectives:

This project has two objectives. The first is to elucidate molecular processes of sperm maturation during transit through the epididymis. Sperm maturation is thought to be associated with the modification of sperm surface glycoproteins which may play critical roles in the initial interaction between sperm and egg during fertilization via highly specialized intercellular recognition and fusion processes. The use of specific monoclonal antibodies against rat sperm surface determinants is employed to identify the binding sites of sperm monoclonal antibodies, any alterations in sperm function, and the origin of sperm surface proteins. The second objective is to determine whether demethylation of 5-me-cytidine of the P1-450 structural gene is associated with the potentiation of 2,3,7,8-tetrachlorodibenzo-P-dioxin-induced transcription of P1-450 mRNA by nucleotide modifiers. Three cDNA probes for the P1-450 gene are used along with selected restriction enzymes to estimate the frequency of demethylation in the P1-450 gene sequence.

Experimental Approach and Scientific Justification:

Determination of the site and origin of sperm surface proteins. Three monoclonal antibodies directed against spatially distinct surface binding sites are used to determine the distribution of specific rat sperm surface antigens, the origin of these proteins, and temporal changes in sperm surface proteins during epididymal transit. These studies are carried out on spermatids and on spermatozoa from testes and precaput, caput, corpus, and cauda epididymides, which are collected and purified by sucrose density gradient centrifugation. Indirect immunofluorescence assays are used to define the sperm surface antigenic sites recognized by three monoclonal antibodies (EA6B, AD8B, and BH9C) directed against the mature rat sperm.

In order to determine the source of sperm surface antigenic determinants, two approaches are used: (1) post embedding immunofluorescence staining of epididymal tissue sections obtained from testes, precaput, caput$_1$, corpus, and

$cauda_2$ epididymides to identify the cell types in different segments, which may react with three sperm surface monoclonal antibodies; and (2) PAGE and immunoblot analysis of epididymal cells isolated from precaput, $caput_1$, $caput_2$, $cauda_1$ and $cauda_2$ epididymides to identify and characterize biochemical properties of these proteins. These experimental approaches will delineate whether the appearance of the antigens on the sperm surface is from the epididymis or is a result of temporal modifications of sperm surface proteins intrinsic to maturing sperm in the epididymal milieu.

To determine whether any of the three spatially distinct mature rat sperm antigens have functional roles in fertilization, assays for the acrosome reaction, motility and fertilization capacity are being developed to test the effects of antibodies on these processes. Enzyme release during the acrosome reaction is determined using BAEE as a substrate. Motility is observed under a phase microscope as a function of time after exposure to monoclonal antibodies. Fertilizing capacity is determined by artificial insemination, using sperm treated with antibodies. These approaches will identify particular sperm surface proteins with putative roles in reproductive processes. Understanding physiological roles of sperm surface proteins which appear or are modified during epididymal maturation will provide a sound basis for studying effects of environmental chemicals on this process.

TCDD-induced potentiation of P_1-450 gene activation. At the present time, the factors that control gene expression in eukaryotic cells have not been well elucidated. Likewise, TCDD-induced potentiation of P_1-450 gene activation by DNA modifiers is not well understood. There appears to be, however, two levels of modification that correlate with gene activity: (1) active genes are in a special chromatin conformation that makes DNA more sensitive to DNAase I; and (2) methylation of DNA nucleotides (5-me-cytidine in eukaryotes) modifies gene activity. By using methylation-sensitive restriction enzymes to probe specific sequences, it has been shown in several systems that the methylation pattern of a particular gene is tissue specific, with a general correlation between gene expression and undermethylation of 5-me-cytidine. In order to determine whether activation of prostatic P_1-450 gene by TCDD and TCDD plus DNA modifiers is correlated with demethylation of P_1-450 genomic sequences, prostate genomic DNA is treated with HPA II and Msp I restriction enzymes and subsequently hybridized with three cDNA probes for the P_1-450 structural gene to assess the extent of demethylation of the prostatic P_1-450 gene. DNA sequencing of the P_1-450 structural gene will be required eventually to determine which sequences are demethylated.

Recent Accomplishments and Significance:

Epididymal sperm monoclonal antibodies. The monoclonal antibodies EA6B, AD8B, and BH9C reacted with different sites on sperm from the precaput, caput, corpus, and cauda epididymides. EA6B reacted equally with the head, surface, and entire tail in precaput sperm. However, as sperm traversed the epididymis, the tail demonstrated a greater fluorescence than the head. On the other hand, AD8B reacted primarily with the entire tail and very weakly with the postacrosomal region, while BH9C reacted exclusively with the midpiece region of sperm from corpus and cauda epididymidis. The origins of these sperm surface

antigens will be determined by immunostaining of epididymal tissue sections and immunoblotting of 2-D gel of extracts of precaput, caput, corpus, and cauda epididymides.

TCDD effect on prostatic genomic DNA. Preliminary results with HPA II and MSP I restriction enzymes demonstrated that both TCDD alone and TCDD plus procarbazine treatment significantly reduced GCC-me-G sites of P_1-450 genes in prostate genomic DNA. Demethylation of P_1-450 gene appeared to be associated with potentiation of TCDD-induced prostatic P_1-450 gene product, aryl hydrocarbon hydroxylase activity. Furthermore, procarbazine plus TCDD caused greater reduction of GCC-me-G sites than that of TCDD alone. Therefore, it appears that greater potentiation of prostatic P_1-450 gene by procarbazine plus TCDD may partly be due to the occurrence of greater demethylation than with TCDD alone.

B. PLANS FOR SUBSEQUENT YEAR

Sperm surface determinants. The functional role of sperm surface proteins synthesized in epididymal epithelial cells will be determined with respect to in vivo fertilization capacity, acrosome reaction, motility, and mitochondrial respirations. The effects of xenobiotic chemicals on the expression of these particular gene products will be determined.

Molecular mechanisms for prostatic AHH P1-450 gene expression. The possible effect that demethylation of 5-me-cytidine might have on potentiation of P-450 gene expression at a transcriptional level is not clear at the present time. Up to two thousand-fold induction of aryl hydrocarbon hydroxylase activity suggests that the 30-fold increase of P-450 mRNA level alone may be insufficient to account for such a dramatic increase of AHH synthesis. One possibility is that procarbazine may affect post-transcriptional levels by methylation of GTP to N^7-me-GTP to provide a greater pool of cap sites for mRNA. It is also possible that methylation of mRNA and/or tRNA may also prolong translation time by increasing affinity of rRNA and mRNA. Further studies with DNA sequencing of the P1-450 structural gene together with HPLC-MS spectral analysis of methylated purines and pyrimidines of DNA, mRNA, rRNA, and tRNA may provide the data to understand a possible mechanism of action at pretranscriptional, post-transcriptional, and transcriptional levels. The various molecular parameters will be studied to understand potentiation of the TCDD-stimulated P-450 structural gene in rat prostate glands. The ultimate objective is not only to understand toxic mechanisms of environmental chemicals in male reproductive organs but also to establish a predictive toxicity based on the structural activity relationships among environmental chemicals. Major portions of these experiments will be continued by collaborators.

PUBLICATIONS

Lee, I.P.: Effects of Environmental Metals on Male Reproduction. In Clarkson, T.W., and Nordberg, G. (Eds.): Reproductive and Developmental Toxicity of Metals. New York, Plenum Press, 1983, pp. 253-278.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70090-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Neuroendocrine and Neurochemical Regulation of Gonadal Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Negro-Vilar	Head, Reprod. Neuroendocrinology Section	LRDT	NIEHS
Others:	C. A. Johnston	Staff Fellow	LRDT	NIEHS
	M. M. Valenca	Visiting Fellow	LRDT	NIEHS
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COOPERATING UNITS *(if any)*

LAB/BRANCH
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Experiments carried out by the Reproductive Neuroendocrinology Section are focused on the cellular and subcellular mechanisms regulating the release of luteinizing hormone-releasing hormone (LHRH) and other hypothalamic peptides that participate in the modulation of pituitary hormone release. Specific studies are designed to elucidate the role of monoaminergic neurotransmitters in the release of LHRH from nerve terminals, the nature of the specific aminergic receptors involved in the neuronal activation that precedes LHRH release, the clarification of the post-receptor events that participate in the peptide-release process, the involvement of arachidonate metabolites in amplifying the response to key neurotransmitters, and the additional role played by other intracellular messengers such as Ca^{+2} and other putative intracellular messengers derived from the metabolism of membrane phospholipids. Other parts of the project are directed to perform an in-depth analysis in vivo of the changes in monoamine turnover and metabolism in discrete brain nuclei that are known to be involved in regulation of gonadal function. Different experimental paradigms are employed, to re-create situations calling for an enhanced (or altered) function of the hypothalamic-pituitary-gonadal axis, such as steroid-feedback manipulations, pregnancy, lactation, estrous cycle, stress, ablation of selected endocrine glands or brain areas, etc. Finally, a group of experiments are directed to evaluate the mechanisms underlying the effects on the reproductive sphere of neonatal neurotoxin treatment, as well as the developmental changes and the role of steroids on certain sexually dimorphic patterns of gonadotropin secretion. The results are integrated to provide a comprehensive hypothesis of the complex, multi-level regulatory mechanism modulating gonadal function.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Reproductive events are regulated by highly complex neuroendocrine mechanisms and several levels of integration (hypothalamic, pituitary, gonadal, and reproductive tract) of these mechanisms are recognized. These key levels of integration, while representing very important physiological sites whereupon regulatory events take place, also constitute target areas where many chemicals, drugs, and toxic compounds act to affect reproductive events. Our laboratory has worked extensively in the last several years to define the intimate cellular and subcellular mechanisms that interact at all levels of integration to modulate reproductive function, and to define the specific mode and site(s) of action of different agents that regulate and/or interfere with reproductive processes.

Objectives:

The primary objectives of this research project are the following:

(1) To understand and clearly define the intimate cellular and subcellular mechanisms involved in the release and function of LHRH, particularly in relation to the sequence of events leading to enhancement of neuronal LHRH release in response to noradrenergic activation. Specific objectives to be achieved include determining the location of the NE neurons involved in LHRH release, examining the possible role and/or interaction that other monoaminergic and peptidergic systems may play on this noradrenergic stimulation, defining the nature of the adrenergic receptor(s) involved in the response, the subsequent involvement of arachidonate metabolites in amplifying the response, and the additional role played by other putative intracellular messages, such as Ca^{+2} and elements derived from the phosphatidylinositol cycle.

(2) To define the intimate cellular interactions between steroids and central monoaminergic systems in the regulation of gonadal function, by exploring specific changes in amine metabolism in specific brain nuclei involved in gonadotropin regulation in the absence of gonadal steroids or following replacement therapy of steroids.

(3) To clarify the neurochemical mechanisms that underlie the development and maturation of the central neuroendocrine response to stress, and to define the role played by individual monoaminergic systems and opioid peptides in the release of the "stress" hormones: ACTH, β-endorphin, and prolactin.

(4) To evaluate the mechanisms underlying the enhanced cellular responsiveness at the hypothalamic and pituitary level that is observed in adult animals after neonatal neurotoxin treatment. Elucidation of these mechanisms may provide some fundamental answers toward our understanding of the factors that regulate cell responsiveness under physiological or pathophysiological conditions.

Experimental Approach and Scientific Justification:

A multifaceted approach including neurochemical, pharmacological, and neuroendocrine procedures is applied to selected in vivo and in vitro paradigms representing key reproductive biological models. Special techniques presently employed include: (1) high pressure liquid chromatography (HPLC) combined with electrochemical detection for the concurrent measurement of femtomol levels of monoamines and amine metabolites in discrete brain nuclei, plasma, cerebrospinal, and other biological fluids; (2) microdissection procedures to isolate selected discrete brain regions; (3) separation of brain peptides in tissues and fluids by HPLC followed by radioimmunoassay (RIA); (4) development and characterization of specific antisera for RIA of newly isolated brain peptides; and (5) short term hypothalamic tissue and pituitary cell cultures for the evaluation of cellular and subcellular mechanisms regulating peptide hormone release. Defining the neuronal systems involved in the regulation of gonadal function and understanding of the complex interplay between steroids, peptides, and amines at the cellular level to effectively modulate gonadotropin secretion require the combination of the above technical approaches. These experimental paradigms will allow us to develop a comprehensive hypothesis of the intimate mechanisms involved in neural peptide release, peptide hormone action, and target cell responsiveness.

Recent Accomplishments and Significance:

(a) Experiments from our laboratory have defined the neurochemical mechanisms involved in the release and function of luteinizing hormone-releasing hormone (LHRH), a hypothalamic decapeptide which represents the prime physiological regulator of pituitary gonadotropin secretion and hence, of gonadal function in animals and humans. In particular, we have developed an in vitro short term culture of (hypothalamic) median eminence fragments, a brain region that contains the richest concentration of LHRH-nerve terminals in the central nervous system. It is from this region that LHRH is secreted into the hypophyseal portal circulation to reach the anterior pituitary and stimulate gonadotropin release. Since this is a closed-circuit system of difficult access, a fact which precludes the constant monitoring of changes in LHRH secretion, our method has provided much needed information on the neurochemical events underlying LHRH release under physiological or pathophysiological conditions. With the use of our in vitro median eminence culture preparation, we have ascertained that catecholamines, and norepinephrine in particular, are the key neurochemical signals that trigger release of the peptide from this area of the brain, and do so by interacting specifically with presynaptic, α-adrenergic receptors located in the median eminence region. Stimulation of these receptors leads to release of LHRH primarily through arachidonate metabolites, in particular prostaglandin-E_2. Arachidonic acid by itself can increase LHRH release, but this effect can be nullified by inhibiting cyclo-oxygenase activity. Although other arachidonic products may also enhance LHRH release (including lypooxygenase and the newly discovered epooxygenase, P-450-dependent products), our results indicate that they are not essential for release of the peptide to occur.

(b) Another important portion of our research efforts in recent years has been dedicated to the study of the neurochemical mechanisms that integrate all the environmental (external) and internal signals that the brain is exposed to during the course of a normal (or abnormal) reproductive event. In particular, our studies have characterized the role of central noradrenergic, serotonergic, and dopaminergic systems as integrators of signals (external or internal) that affect pituitary hormone secretion and gonadal function. We have looked extensively at the effects of steroids, in either physiological or pharmacological paradigms, upon the reproductive system and, in particular, within the brain-pituitary axis. Our studies have indicated that the gonadal steroids (estradiol, progesterone, testosterone) can have profound effects upon central monoaminergic and peptidergic systems involved in reproductive function. These effects were characterized during the normal course of reproductive life, and after endogenous or exogenous manipulations were introduced to modify steroid levels in plasma and target tissues. Our studies indicate, for instance, that estrogens can profoundly affect brain regulation of gonadal function by interacting to a large extent with noradrenergic neurotransmitter systems within the preoptic-hypothalamic region of the brain as well as 5-HT systems in the mediobasal hypothalamus. Other steroids, such as progesterone, also can interfere with gonadal function by acting at specific brain sites, but only after specific progestin receptors are induced by prior estrogen priming. These studies have provided a regional mapping within the brain of the cellular and subcellular mechanisms involved in steroid hormone action. Recent additional studies in our laboratory have taken advantage of the information obtained in normal animals to use it in the analysis of the mechanisms mediating the adverse reproductive effects of certain neurotoxins. The data indicate that toxic neurochemicals given during the neonatal period (when the brain is still undergoing key developmental changes) can interfere with the steroid-monoamine-peptidergic neuronal interaction and thereby permanently alter normal gonadal function.

(c) A great number of studies over the past several years have indicated that different stressors can negatively affect reproductive function. Recent developments including the discovery, isolation, and synthesis of a number of hypothalamic peptides involved in the stress response have allowed us to analyze in more detail the mechanisms through which central peptidergic and monoaminergic systems can affect hormone release and, eventually, reproductive functions. In particular, we have initiated studies to evaluate the role of proopiomelanocortin (POMC)-derived peptides such as β-endorphin and ACTH in the stress response, and also the participation of other peptidergic systems involved in the release of the above peptides, namely corticotropin-releasing factor (CRF), vasopressin, oxytocin, etc. This complex regulatory system consists of multiple mechanisms involving peptides that act through specific membrane receptors that we and others have described, and also interactions with brain monoaminergic systems that represent an essential component of the stress response.

B. PLANS FOR SUBSEQUENT YEAR

(a) With regard to the mechanisms regulating LHRH release, additional experiments are needed in this area to clarify the following:

(1) The exact mechanism by which noradrenergic activation of α-adrenergic receptors affect prostaglandin-E_2 and LHRH release. Specific questions to be asked include whether α-1 or α-2 type receptors are involved. This, in turn, will clarify which intracellular mediator (Ca^{+2} or cyclic nucleotides) is more likely to be involved in the action of the catecholamine within this particular neuronal system.

(2) Transmembrane events involved in peptide release. Our studies will focus on the role of stimulated phosphatidylinositol (PI) turnover and protein phosphorylation on the release of peptides. Studies will be conducted to evaluate the possibility of measuring inositol phosphates formation as well as protein phosphorylation in our tissue preparation in vitro, after either in vivo or in vitro treatment with specific hormone secretagogues.

(3) Pertaining to the previous item, we will continue our studies which have characterized the role of calcium channels and of intracellular Ca^{+2} release on the stimulation of LHRH induced by membrane depolarizing agents or by prostaglandins, respectively. Experiments will be performed to evaluate the exact role of intracellular Ca^{+2} on peptide release, using newly developed probes (such as quin-2) for the quantitation of intracellular Ca^{+2} release after stimulation with selected agents. In conjunction with the studies outlined above, these observations should provide much needed information concerning the molecular mechanisms involved in the regulation of peptide release and hormone action.

(b) In the area of neurotoxin-induced hypothalamic dysfunction, additional studies will be conducted to further define the role of other important chemical messengers. Moreover, and in view of very recent studies from our laboratory that indicate a generalized increase in the magnitude of membrane and/or transmembrane events leading to peptide secretion after chronic neurotoxic damage, we intend to analyze the possible mechanism(s) mediating this intriguing phenomenon. In continuation of earlier work in which we showed that estrogens can affect certain parameters of brain sexual differentiation during the neonatal or adult periods, we will perform experiments to determine the neurochemical correlates of estrogen action within the brain during the neonatal period.

Finally, studies will be developed to analyze in detail the maturation of the stress response during development. Since, according to the age of the animal, different hormones are progressively incorporated to the neuroendocrine response to stress, we will take advantage of this natural sequence of events to dissect the role of the individual systems involved in the release of each stress-responsive hormone. The deleterious effects of elevated prolactin levels upon reproductive functions are very well known, and hyperprolactinemia is one of the most frequently cited causes of human infertility of endocrine origin. In line with these observations, our studies on stress and neurochemical regulation of hormone release will also provide additional and needed information concerning the role, interactions, and connectivity of endogenous opiate, serotonergic, and dopaminergic systems in the regulation of prolactin release under different experimental conditions during development and later in life. This intimate dissection of the neural sequence of events mediating

ACTH, β-endorphin, prolactin and growth hormone release under normal and stressful conditions may also provide relevant information concerning the usefulness of different psychoactive compounds to treat or prevent stress-mediated events. Finally, the results may also shed light on the mechanism(s) of action of environmental, toxic or pharmaceutical chemicals and psychoactive drugs that can profoundly affect reproductive functions when received accidentally or for therapeutic reasons.

PUBLICATIONS

Negro-Vilar, A.: Mechanisms Regulating LHRH Release: Implications For Fertility Regulation. In Harrison, R. and Thompson, J.B.W. (Eds.): Fertility and Sterility. Lancaster, England, MTP Press, 1984, pp. 15-23.

Johnston, C.A., Spinedi, E., and Negro-Vilar, A.: Aromatic L-amino acid decarboxylase activity in the rat median eminence, neurointermediate lobe, and anterior lobe of the pituitary: physiological and pharmacological implications for pituitary regulation. Neuroendocrinology (In Press).

Spinedi, E., Johnston, C.A., and Negro-Vilar, A.: Increased responsiveness of the hypothalamic-pituitary axis after neurotoxin-induced hypothalamic denervation. Endocrinology (In Press).

Johnston, C.A., Spinedi, E., and Negro-Vilar, A.: Effect of acute ether stress on monoamine metabolism in median eminence and discrete hypothalamic nuclei of the rat brain and on anterior pituitary hormone secretion. Neuroendocrinology (In Press).

Negro-Vilar, A: Molecular mechanisms regulating LHRH release. Proceedings of Symposium on Endocrine Pharmacology, Acta Physiol. Pharmacol., (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 70092-01 LRDT

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cellular and Molecular Mechanisms Mediating Peptide Hormone Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Negro-Vilar	Head, Reprod. Neuroendocrinology Section	LRDT NIEHS
Others:	E. Spinedi	Visiting Associate	LRDT NIEHS
	C. A. Johnston	Staff Fellow	LRDT NIEHS
	M. M. Valenca	Visiting Fellow	LRDT NIEHS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Reproductive and Developmental Toxicology

SECTION
Reproductive Neuroendocrinology Section

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.9	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Analysis of the cellular and molecular mechanisms mediating peptide hormone action constitute an important component of the research efforts of the Reproductive Neuroendocrinology Section. The close interrelationships mediating neuroendocrine responses within the hypothalamic-pituitary-gonadal systems offer an excellent opportunity to analyze some unique characteristics of peptide-peptide, peptide-amine, and peptide-amine-steroid interactions. Studies using pituitary cell cultures are directed to evaluate the precise mechanisms through which peptidergic or aminergic secretagogues enhance or suppress peptide hormone release. Protocols are designed to evaluate characteristics of hormone-receptor interactions, post-receptor as well as transmembrane events involved in the hormone-release process, and definition of the specific intracellular messengers transducing the action of key hypothalamic peptides involved in pituitary hormone release. Other studies are designed to explore the intriguing peptide-amine interactions (such as serotonin-AVP) previously reported by our group, to better understand the nature and physiological significance of those interactions.

At the testicular level, studies are designed to determine the intratesticular effects of LHRH-analogs, known to adversely affect both the endocrine and the gametogenic functions of the testis. Since some of these analogs are presently being tested for use in human contraception, an understanding of their site(s) and mechanisms of action is of obvious significance. The interaction of these LHRH-A with intrinsic peptidergic systems within the testis, such as the proopiomelanocortin-derived peptides, is also being explored. The results may provide very significant advances to our knowledge of paracrine and/or autocrine effects of gonadal peptides.

PROJECT DESCRIPTION

A. RESEARCH PROJECT

Nature of Problem:

Great advances have been made in recent years to further our understanding of some of the intrinsic mechanisms of hormone action. Several basic systems of intracellular messengers coupled with membrane receptors or of cytosolic receptors which, when translocated to the nucleus, affect the genome have been defined in a variety of systems. Within the hypothalamic-pituitary-gonadal system, a diversity of aminergic, peptidergic, and steroidal hormones exert profound hormonal effects by invoking a multitude of cellular responses. In each individual case, the hormone-cell interaction acquires special characteristics according to the chemical nature of the hormone involved, and to the type and location of the target cell. Most hormones exert specific effects at selected sites, and this specificity usually involves stimulation (or sometimes inhibition) of key intracellular messengers after interaction with a specific cellular receptor. Definition of the sequence and type of events mediating the effects of different peptides or amines within the hypothalamic-pituitary-gonadal axis is, therefore, of paramount importance toward our understanding the intimate cellular and subcellular mechanisms mediating major reproductive events.

Objectives:

The primary objectives of this research project are the following:

(1) To evaluate and characterize the mechanism(s) and specific cellular and subcellular site(s) of action of certain key peptidergic and aminergic neurohormones known to be secreted from the hypothalamus into the portal circulation and which modulate the release of different pituitary hormones clearly involved in reproductive functions. In addition, special attention will be given to peptide-amine interactions that may well represent an important mode of combined hormonal regulation. Finally, possible paracrine and autocrine actions of peptide hormones will also be evaluated.

(2) To determine the presence and characterize the identity and location, within specific compartments or cell types of the testis, of several peptidergic factors which have been recently postulated to be present in the testis of several species. These studies will, in addition, attempt to define the physiological role of these peptides, as well as the particular circumstances in which their action is clearly expressed.

Experimental Approach and Scientific Justification:

Most of the studies will require, by necessity, the use of *in vitro* systems, and a combination of either short-term or long-term tissue or cell cultures. In the case of the pituitary gland, and due to the heterogeneity of the cell types, attempts will be made to submit enzymatically dispersed cells to an elutriation procedure which may provide useful enrichment of certain cell

types. In addition, cell cultures will be submitted to specific stimulation by hypothalamic peptide hormones, and attempts will be made to mimic the pulsatile rate of delivery of these neural hormones with the use of recently developed computerized cellular engineering perfusion systems. Intracellular uptake and processing of peptides and amines will be followed by autoradiography. After selective stimulation of hormonal release, activation of selected intracellular messengers (Ca^{+2}, cyclic nucleotides, elements of the PI cycle, etc.) will be evaluated.

At the testicular level, our studies will be directed to determine the presence, mechanism, and site of action of specific peptides, such as β-endorphin, ACTH, arginine vasopressin (AVP), luteinizing hormone releasing hormone (LHRH), etc. To determine the changes in secretion rates of these peptides from the different testicular cell compartments, collection of testicular interstitial fluid will be performed using techniques already available in our Section, after certain experimental manipulations are performed to either enhance or suppress the endocrine and/or gametogenic functions of the testis. Moreover, some of the above peptides will be microinjected into the interstitial compartment of the testis to evaluate their effects and site of action under different physiological situations. Measurements of peptide levels will be made by sensitive and specific radioimmunoassays after appropriate purification by HPLC. Selective blockade of peptide action will be attempted by the use of specific receptor blockers or by employing selected peptide antagonists currently available.

Taken together, the above studies will provide important answers toward our better understanding of peptide hormone action, particularly in the area of receptor activation, transmembrane events leading to hormone release, role of specific intracellular messengers in hormone output, peptide amine interactions, autocrine and paracrine peptide actions, and expression of peptides in reproductive tissues.

Recent Accomplishments and Significance:

(1) Work carried out by scientists presently at the Reproductive Neuroendocrinology Section has established and characterized specific pituitary membrane receptors for hypothalamic peptides such as angiotensin II (AII) and arginine vasopressin (AVP). In both cases, high affinity, low capacity membrane receptors were identified, with K_d's in the low nanomolar range, values found compatible with the doses of peptide required to elicit a normal physiological response. In the case of AVP, the use of ^{3}H-ligand enabled us to evaluate both the binding characteristics as well as the biological activity (enhanced ACTH and β-endorphin release) using intact, dispersed anterior pituitary cells. Specific receptor blockers, like saralasin, blocked the action of AII, and other agonists of either AII or AVP showed binding affinities comparable with their respective biological activities. Since other groups have shown that corticotropin releasing factor (CRF) acts also on specific membrane receptors, it is clear that three different peptides that stimulate ACTH/β-endorphin secretion do so by acting on specific and independent receptors. Moreover, while CRF-stimulated ACTH release involves cAMP activation, the effect of AVP

is not mediated by activation of that nucleotide, strongly suggesting that other intracellular messengers might be involved in the action of AVP and, perhaps, of AII.

Additional studies from our group have clearly established an intriguing peptide-amine interaction between AVP and serotonin to enhance ACTH release from the pituitary. Several additional studies involving the use of specific serotonin receptor blockers, serotonin synthesis inhibitors, or tissues obtained from rats with a genetic deficiency in AVP synthesis, indicate that the AVP-serotonin interaction can be observed in vivo and might, therefore, constitute an important physiological mechanism in the regulation of ACTH release.

(2) Recent studies in our Section have established the presence of proopiomelanocortin (POMC)- derived peptides in testicular interstitial fluid (TIF). Using highly sensitive and specific radioimmunoassays, we established the presence of β-endorphin and ACTH in TIF from both intact and hypophysectomized animals. The latter observation clearly indicates that the peptides found in TIF are not derived from the peripheral plasma, since after removal of the pituitary gland plasma levels of both POMC-peptides are undetectable. Authenticity of the peptides measured was further established by chromatography and parallelism of serial dilutions with the standard curve in the radioimmunoassays. Recent experiments indicate that stimulation with either LH or HCG both in intact or hypophysectomized animals can profoundly affect levels of these peptides in TIF suggesting a relationship between the gonadotropin and the POMC-derived peptides.

Other studies have been conducted to elucidate the effects of superactive LHRH analogs on testicular function. Chronic and continuous administration of the LHRH-A has been shown to suppress both the endocrine and gametogenic functions of the testis, representing the basis for the potential use of these compounds as contraceptive agents. Studies in the Reproductive Neuroendocrinology Section have been directed at analyzing the direct intratesticular effects of these analogs of LHRH on testosterone secretion and also their possible interactions with other peptidergic systems present in the testis. Preliminary results indicate that an interaction between LHRH and POMC-derived peptides in the testis may be involved in the regulation of testosterone secretion.

B. PLANS FOR SUBSEQUENT YEAR

Several experimental approaches will be employed to evaluate peptide/amine effects at the cellular/subcellular level in the anterior pituitary, including:

(1) Role of specific intracellular messengers on the peptide-induced anterior pituitary hormone release: using specific and selected peptidergic secretagogues, such as AVP, AII, CRF, LHRH, or TRH, attempts will be made to characterize the cellular events leading to the exocytotic secretion of hormones. Particular attention will be given to the possible effects of peptides on membrane phospholipids. This will be accomplished by using P-32 labeled cells to study the breakdown and resynthesis of phosphatidylinositol, by measuring activation of the phospholipid-dependent protein kinase C, and by employing

certain specific probes such as diacylglycerol, phorbol esters, and ionophores. Since either inositol biphosphate (IP_2) or inositol triphosphate (IP_3) may function as messenger(s) to transmit the signal expressed by surface receptors to the interior of the cell, we will explore the possibility that one of these factors may be involved in the action of some of the above peptides. In addition, and in light of recent evidence that we and others have provided indicating that arachidonic acid and/or arachidonate metabolites can enhance pituitary hormone release, experiments will be conducted to evaluate the linkage between the receptors regulating arachidonic acid turnover and the PI cycle. The role of Ca^{+2} on the above events will similarly be explored.

(2) Peptide-amine and peptide-peptide interactions at the anterior pituitary level will be analyzed using cell cultures of dispersed anterior pituitary cells and cell separation techniques. Combined stimulation with peptides using selected pulsatile delivery patterns in a continuous perifusion system will be employed to evaluate peptide-peptide interactions. Similar approaches will be used to analyze peptide-amine interactions. Cells will be obtained from animal models in which the secretion of the appropriate pituitary hormone is selectively enhanced or suppressed, or after experimental manipulations designed to alter the in vivo input of a given peptide or amine. When appropriate, autoradiographic studies will be performed to analyze the uptake and intracellular handling of peptidergic or aminergic ligands.

(3) Recently developed superagonists of LHRH, currently being evaluated for clinical use in contraception, will be employed to determine the direct effects at the testicular level as well as possible indirect effects at central (hypothalamic) and pituitary levels. Specific parameters to be evaluated at the testicular level will be testosterone secretion into testicular interstitial fluid and peripheral plasma, as well as testosterone secretion from whole testis in short-term in vitro cultures. The possible interaction between LHRH-A and other testicular peptides, particularly β-endorphin and ACTH will also be evaluated. In selected experiments, blockade of opioid receptors will be achieved by naloxone or naltrexone pretreatment, to determine the role of endogenous testicular opioids on the endocrine function of the testis.

PUBLICATIONS

Spinedi, E. and Negro-Vilar, A.: Arginine vasopressin and ACTH release: correlation between binding characteristics and biological activity in anterior pituitary dispersed cells. Endocrinology 114: 2247-2251 (1984).

Negro-Vilar, A., Spinedi, E., and Johnston, C.A.: Monoamine Peptide Interactions in the Regulation of Pituitary Cell Function. In Labrie, F. (Ed.): Proceedings of the Seventh International Congress of Endocrinology. Amsterdam, The Netherlands, Elsevier, (In Press).

Negro-Vilar, A. and Valenca, M.: Testicular Peptides Regulating Gonadal Function. In Sairam, M. (Ed.): Gonadal Proteins and Peptides and Their Biological Significance. Singapore, Republic of Singapore, World Science Publishing Company, (In Press).

COMPARATIVE MEDICINE BRANCH

COMPARATIVE MEDICINE BRANCH
Summary Statement

Administrative Activities

CMB activities had important impact on NIEHS operations during 1984. Glassware and Media Preparation facilities on the North Campus were closed and moved to Building 101, South Campus, in January. This move was executed with less than a one week shut down of service and no shut down of high priority needs. The Animal Facilities of the C & D Modules, Building 101, were occupied in September 1983. Most mechanical difficulties inherent in the occupation of the new space were anticipated well enough in advance to avoid major problems. Nevertheless, the occupation of these facilities forced solutions to problems in climate control systems, distributed ultrafiltered water systems, pneumatic materials movement systems, service equipment, communications systems, security systems, etc. Failure of an ultrafiltered water system pump resulted in contamination of this system with pump lubricant, and caused the only emergency situation experienced so far. An alternate deionized water supply was installed within days of the contamination. This system supplies drinking water to animals and ultra processed water to glassware cleaning equipment and media preparation.

Other major activities included: 1) The start of Building 15 (North Campus) remodeling, scheduled for completion in December 1984. This will relocate LBNT behavior laboratories to modern space, and completely remodel the service area for Building 15, including new washing machines. 2) Closure of the Duke Rodent Facility as scheduled. All personnel, equipment, and services were relocated to D Module, Building 101. 3) Full operation of the Remote Isolation Unit (environmental room), and the opening of a second unit, to accommodate special investigator needs to work with animals from sources of unknown quality; however, environment control in these units remains a problem. 4) The institution of the use of Ventilated Cage Racks for containment of toxic substances studies within existing standard animal space. This system may also be useful for disease control and reducing energy demands because of concentrated pathways of air distribution. However, an important gap remains in the process of handling animals exposed to toxic substances that this system does not address. This problem will be addressed during the next year. 5) Activation of a Technical Assistance Program to train scientists and technicians in methods of animal handling, parenteral techniques, restraint, surgery, specimen collection, etc. This program is not intended as a technical service program, but is intended to meet special requirements to facilitate experiments and train personnel, and to enhance the Institute's ability to deal with questions of humane care and treatment of experimental animals.

During 1984 the Animal Husbandry Section was reorganized to include a supervisor trainee position and an Animal Facilities Manager with overall responsibility for daily operation of Institute animal facilities. A Staff Assistant position was created in the CMB office to address responsibilities in planning, training, resource estimation, Project Plan review as required by NIH for animal experiments, and other staff responsibilities. An organization assessment of the Glassware Processing facility was accomplished through outside professional services. This assessment was completed in preparation for contracting the Glassware Processing function to an outside organization. Procedures in Glassware Processing and Media Preparation were reviewed internally and important

modifications in operating procedures and quality assurance methods will be made. Major new emphasis has been placed on Quality Assurance responsibility within CMB. This effort was given separate stature with responsibility for quality assurance of animals acquired for research, feed, water, biological media, biologicals for use in animals, and related administrative problems.

Major emphasis continues in rodent disease control and investigation. While colony health has improved, the colony remains vulnerable to disease problems in supplier colonies. Goals for 1985 include the opening of Building 101, E Module Animal Facility, restricted to selected studies. Plans and preparations are in progress. Reopening of Building 15, and reassessment of the division of labor within CMB to meet demands and improve organization structure are also major objectives.

Research Activities

Research activity of the Laboratory Section of CMB was aimed at specific problems affecting Institute research animals. Evaluation of laboratory animal diet for estrogenic substances using the standard mouse bioassay showed that this test may not be reliable unless carefully controlled. Statistical examination of assays conducted by contract laboratories showed that in 3/9 assays control uterine weight : test uterine weight ratios did not meet valid assay criteria. Examination of uterine weight : body weight ratios showed unacceptable variation unless test mouse age is carefully controlled at the start of the assay. Experiments to establish the lower and upper limits of age acceptable for a successful bioassay have been completed. Lower age is limited by the age at which mice can be successfully weaned (14-16 days); upper age by the first onset of endogenous hormone activity, after which uterine weight increases rapidly. Variability in uterine weight increases begins as early as 22 days; thus, only a narrow window of useful starting age exists because of the length of the assay (7 days). Further tests are now in progress to determine the lowest level of diethylstilbesterol in a semi-purified diet that is detectable.

Additional research activity focused on disease of laboratory animals. The potential effects of the anthelminthic trichlorfon (O,O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate) on the immune system of the mouse were studied by measuring splenic plaque-forming cells; delayed hypersensitivity to keyhole limpet hemocyanin (KLH); lymphoproliferative response to mitogens (B-cell-*E. coli* lipopoly saccharide; T-cell-Concanavalin A and phytohemaglutinin; one way mixed leukocyte cultures - mitomycin-C); and organ/body weights. Aside from absolute increases in liver and kidney weights, and liver : body and kidney/body in treated animals, no other parameters differed significantly. It was concluded that treatment of mice with trichlorfon does not affect typical immunological parameters. Studies on rabbit coronavirus (RbCV) showed that specific hematological values fall rapidly following experimental infection, and that blood platelet counts may be a predictor of death. Coronaviruses currently rank as one of the most important adventitious influences on research involving rodents. Further attempts will be made to investigate the relatedness of RbCV to other, more important, rodent coronaviruses (MHV, RCV), and how relatedness might be used to modify or prevent infections by these viruses which have an important impact on experimental rodent health.

Collaborative efforts of a non-project nature continue between Oak Ridge Associated Universities (ORAU) and CMB on investigation of colon cancer and reproductive biology in the cotton-top tamarin (Saguinus oedipus oedipus). This primate experiences high (30-50%) incidences of naturally occurring colorectal carcinoma and inflammatory bowel disease (IBD), possibly due to environmental or nutritional influences. Efforts to propagate this endangered species in captivity to preserve the usefulness of the model appear to have been successful. Second generation infant rearing in captivity now occurs with regularity at the ORAU facility. Early life behavioral imprint centering on care of younger siblings is essential to successful maternal behavior later. Cancer incidence in colony-born individuals parallels the incidence rate in jungle born individuals. The relationship of IBD to colorectal carcinoma in this species is uncertain. Both topics were discussed at length at the workshop at ORAU in April 1984 (Is the Marmoset an Experimental Model for the Study of Gastrointestinal Disease?). Specimens collected from jungle animals immediately after trapping will be examined to determine if IBD is a natural or environment/nutritionally induced phenomenon in captivity.

Goals for 1985 include further investigation into the biology of MHV and related viruses, completion and publication of a revision for the mouse bioassay for estrogenic substances, and investigation into methods of rodent corona virus control, possible through vaccination. Collaborative efforts with ORAU in the field of tamarin colorectal carcinoma and IBD will continue with efforts to investigate genetic control of cancer expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 22102-03 CMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of a Coronavirus from Rabbits

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. D. Small	Head, Diagnostic Laboratory	CMB, NIEHS
Others:	M. E. Clements	Bio. Lab. Tech.	CMB, NIEHS

COOPERATING UNITS *(if any)*
Chemical Pathology Branch, TRTP, NIEHS, NIH (Dr. Thompson); Division of Comparative Medicine, Johns Hopkins School of Medicine (Drs. J. Strandberg and L. Aurelian)

LAB/BRANCH
Comparative Medicine Branch

SECTION
Diagnostic Laboratory

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to study the pathogenesis of rabbit coronavirus (RbCV), the rabbit's physiologic response to this virus, and the relatedness of RbCV to other members of the Coronaviridae. Pleural effusion disease (PED) virus and RbCV have been shown to induce similar clinical signs and lesions. Each virus induces antibody which partially neutralizes both viruses. Blood from rabbits given RbCV exhibited decreased platelet counts, RBCs, hematocrits, and hemoglobin levels. Leukopenia followed by leukocytosis with a left shift was seen. Serum creatine kinase (CK) levels increased as much as 217 times pre-inoculation levels on day 4 post infection. With the elevated CK, lipemia was common. Prothrombin time increased from 9.1 seconds to greater than 150 seconds 4 days following infection.

Assessment of myocardial damage using ECGs and creatine kinase isozymes are beginning. Likewise, further examination of coagulation factors, activated partial thromboplastin time, prothrombin time, thrombin time and fibrinogen levels are planned. Antibodies to other coronaviruses are being produced for use in studying the relatedness of RbCV to other Coronaviridae.

The significance of this work lies in the ability to study a viral disease with a cardiotropism in an animal of sufficient but manageable size to allow sequential clinical and physiological observations. The damage to the rabbit heart by RbCV has a corollary in the human heart with the Coxsackie viruses, Mycoplasma pneumoniae, influenza virus, Herpes zoster, and possibly other infectious agents.

PROJECT DESCRIPTION

METHODS EMPLOYED: Inoculation of rabbits and collection of blood was by previously reported procedures. Hematologic assays were done on an ORTHO ELT-8/ds Hematology Analyzer. Clotting values were determined with a General Diagnostics COAG-A-MATE. Creatine kinase levels were determined with a Centrifi Chem using NAD reduction according to the method of Rosalki. Antibodies to calf diarrhea coronavirus and transmissible gastroenteritis virus (a porcine virus) are being made in rabbits using commercially available vaccine.

MAJOR FINDINGS AND PROPOSED COURSE: Blood from rabbits given rabbit coronavirus (RbCV) exhibited decreased platelet counts, RBCs, hematocrits, and hemoglobin levels. Leukopenia followed by leukocytosis with a left shift was seen. Serum creatine kinase (CK) levels increased as much as 217 times pre-inoculation levels on day 4 post-infection. With the elevated CK, lipemia was common. Prothrombin time increased from 9.1 seconds to greater than 150 seconds 4 days following infection. Assessment of myocardial damage using ECGs and CK isozymes are beginning. Likewise, further examination of coagulation factors, activated partial thromboplastin time, prothrombin time, thrombin time and fibrinogen levels are planned. Antibodies to other coronaviruses are being produced for use in studying the relatedness of RbCV to other Coronaviridae.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The coronaviruses are a large group of species specific viruses which frequently share common antigens. They present a variety of clinical manifestations, due to enteric, respiratory, and neurologic tropisms. RbCV is the first coronavirus identified with a myocardial tropism. The significance of this lies in the ability to study a viral disease with a cardiotropism in an animal of sufficient but manageable size to allow sequential clinical and physiologic observations. The damage to the rabbit heart by RbCV has a corollary in the human heart with the Coxsackie viruses, Mycoplasma pneumoniae, influenza virus, Herpes zoster, and possibly other infectious agents.

The study of the Coronaviridae in the broader scope has value because in mice and rats these viruses (MHV and RCV/SDA) are responsible for the majority of morbidity in NIEHS animals as well as in animals of other institutions. This morbidity has been responsible for the loss of several projects in recent years resulting in a loss of time and money. A greater understanding of the biology of the coronaviruses and the development of vaccines to those of the mouse and rat would be most helpful.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 22104-01 CMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Assessment of the Mouse Bioassay Test for Detecting Estrogenic Activity in Feed

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. E. Thigpen	Microbiologist	CMB, NIEHS
Others:	C. B. Richter	Vet. Med. Ofcr.	CMB, NIEHS
	L. A. Li	Biostatistician	BRAP, NIEHS
	J. K. Haseman	Biostatistician	BRAP, NIEHS
	C. W. Jameson	Chemist	TRTP, NIEHS

COOPERATING UNITS *(if any)*
Animal Husbandry

LAB/BRANCH
Comparative Medicine Branch

SECTION
Quality Assurance Laboratory

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.8	.3	.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of this project were to evaluate and make recommendations for improving the mouse bioassay (NIH, Std. No. 2C, 12-8-72) for detecting estrogenic activity in animal diets. The test diets from 9 of 9 bioassays were reported to be non-estrogenic by a contract testing laboratory. When results were analyzed, 3 of 9 bioassays were determined to be invalid. The mean uterine weight to the terminal body weight ratio for the positive control mice was not 1.5 times as great as that of the negative control mive, as required for a valid bioassay. Uterine weights for the negative control mice of the 3 invalid bioassays were significantly heavier (P less than .01) than the uterine weights for the negative control mice of the 6 valid bioassays. These higher uterine weights in some of the negative control mice may have been due to endogenous hormone activity, probably as a result of failure to control test animal age within the recommended range (14-17 days). Uterine weight increases caused by hormonal activity may occur as early as 22 days of age in some female mice; hence, bioassay studies should be concluded before uterine weight increases due to endogenous hormone begin. To establish the lower limit of useable age, female mice were weaned at 14, 16, 18, or 20 days of age and body growth curves determined. Mice weaned at 14 days and weighed 2 days later lost weight; however, by 24 days of age, they were indistinguishable from mice weaned at 16, 18, or 20 days. Female mice weaned at 16 days of age were chosen to determine the body : uterine weight ratio growth curves. These females showed increases with minor variations in weights from 16-22 days of age. After 22 days of age rapid uterine growth occurred in some mice resulting in large variations in uterine weights from 22-32 days. These results suggest that data collected beyond the 22 day age are misleading in terms of the bioassay, and that only carefully controlled age ranges of weanling mice are suitable for the bioassay.

PROJECT DESCRIPTION

METHODS EMPLOYED: Shipments of NIH-31 feed were analyzed for estrogenic activity using the mouse bioassay by a contract laboratory in accordance with NIH Standard No. 2C, 12-8-72. Assays were considered invalid when the mean uterine weight to terminal body weight ratio for the mice fed a positive control (estrogenic) ration was not at least 1.5 times as great as the ratio for the negative control ration. Pups from female mice with standardized litters were weaned at 14, 16, 18, and 20 days of age and body weight, uterine weight and body : uterine weight ratios were established through 32 days of age. These data were examined using parametric methods. These studies are being repeated using a semi-purified diet (AIN-76, modified). Finally, semi-purified diet was spiked with 0, 2, 4, 5 or 6 ppb DES and examined by gas chromatography for confirmation.

MAJOR FINDINGS AND PROPOSED COURSE: Test diets from 9 of 9 bioassays were reported to be non-estrogenic by a contract testing laboratory. When results were analyzed, 3 of 9 bioassays were determined to be invalid. The mean uterine weight to the terminal body weight ratio for the positive control mice was not 1.5 times as great as that of the negative control mice, as required for a valid bioassay. Uterine weights for the negative control mice of the 3 invalid bioassays were significantly heavier (P less than .01) than the uterine weights for the negative control mice of the 6 valid bioassays. These higher uterine weights in some of the negative control mice may have been due to endogenous hormone activity, probably as a result of failure to control test animal age within the recommended range (14-17 days). Some female mice experience first estrus at approximately 25 days of age, and at this time uterine growth is rapid; hence, bioassay studies should be concluded before female mice begin estrus. To establish the lower limit of useable age, female mice were weaned at 14, 16, 18, or 20 days of age and body growth curves determined. Mice weaned at 14 days and weighed 2 days later lost weight, but later recovered to equal weights of mice weaned at 16, 18, or 20 days. Body and uterine weight growth curves for female mice weaned at 16 days of age showed increases with minor variations in weights from 16-22 days of age. In contrast, rapid uterine growth occurred in some mice after 22 days of age, resulting in large variations in uterine weights from 22-32 days. These results suggest that only narrow, carefully controlled age ranges of weanling mice are suitable for the bioassay. Present studies will compare growth curves/uterine weights of weanling females on a semi-purified diet (AIN-76, modified), to females on standard laboratory diets. Next, we plan to determine uterine weight growth curves at 2 day intervals from 16-28 days of age while feeding a semi-purified diet (AIN-76, modified) and/or standard lab. diet (Purina #5002) containing 0, 2, 4, 5 or 6 ppb of DES. At that point we anticipate that necessary parameters for proper conduct of the mouse bioassay for estrogenic substances will have been more carefully defined.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Naturally occurring plant products, pesticides, feed additives, and some related steroids may exhibit estrogenic activity when consumed by domestic and laboratory animals. The principal activity of NIEHS involves biomedical research effects of environmental agents on human health. Research at NIEHS includes

extensive studies in reproductive and developmental toxicology; hence, substances exhibiting estrogenic activity in animal feeds are undesirable. The wide range of potentially active substances renders specific analysis impractical and uneconomical. The mouse bioassay remains the most useful test to detect estrogenic activity in animal diets, but must be carefully controlled.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 ES 22105-01 CMB

PERIOD COVERED
October 1, 1983 to September 30, 1984

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Trichlorfon on the Immune System of the Mouse

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. D. Small	Vet. Med. Ofcr.	CMB, NIEHS
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	M. E. Clements	Bio. Lab. Tech.	CMB, NIEHS
	E. H. Lebetkin	Bio. Lab. Tech.	CMB, NIEHS

COOPERATING UNITS *(if any)*
Systemic Toxicology Branch, TRTP, NIEHS

LAB/BRANCH
Comparative Medicine Branch

SECTION
Diagnostic Laboratory

INSTITUTE AND LOCATION
NIEHS, NIH, Research Triangle Park, North Carolina 27709

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Trichlorfon, o,o-dimethyl 2,2,2-trichloro-1-hydroxyethyl-phosphonate, an organophosphate pesticide and vermifuge has been used to treat laboratory mice, rats, and hamsters infected with the pinworm, Syphacia obvelata. A concern was the effect of this drug on the animal's immune system. The immunotoxicity of this drug was examined in B6C3F1 mice. IgM antibody production against sheep red blood cells, delayed hypersensitivity to keyhole limpet hemocyanin, and the lymphoproliferative response to E. coli lipopolysaccharide were used to assay B cell function. T cell function was measured by responses to concanvalin A and phytohemagglutinin. No significant effects were observed using the above criteria. A statistically significant increase in liver and kidney but not spleen weight and organ to body weight ratio was observed. These weight differences are thought to be due to the decreased water intake in the drug treated group.

The significance of this project lies in the fact that trichlorfon kills the parasite and is more efficacious than the more commonly used alternative, piperazine, which does not kill the parasite. This data suggests that the use of trichlorfon in mice will not compromise the host's immune system. The increased liver and kidney weights will be examined in a second group of mice.

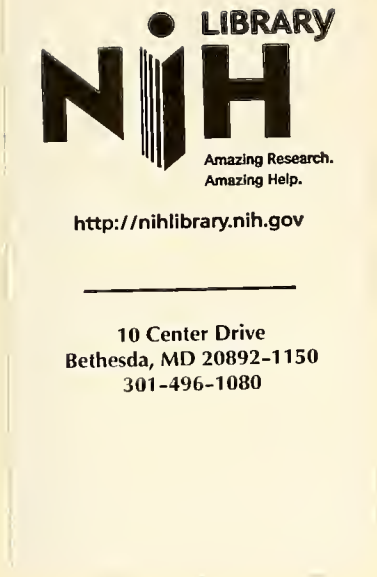
PROJECT DESCRIPTION

METHODS EMPLOYED: B cell function was examined by measuring IgM antibody production against sheep red blood cells, delayed hypersensitivity response to keyhole limpet hemocyanin, and the lymphoproliferative response to E. coli lipopolysaccharide. T cell function was measured by response to concanavalin A and phytohemagglutinin. All assays were done in B6C3F1 mice. Mice were given trichlorfon in the drinking water and water consumption measured.

MAJOR FINDINGS AND PROPOSED COURSE: No significant effects were found using the above criteria. However, a slight yet statistically significant increase in liver and kidney weights and organ to body weight ratio was observed. Additional mice will be used to examine these differences. Weight, feed consumption, water intake, and organ weights will be measured. Tissues will be examined for histologic changes.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Trichlorfon, o,o-dimethyl 2,2,2-trichloro-1-hydroxyethyl-phosphonate, is used to treat laboratory rodents for infections with pinworms, Syphacia spp. and Aspicularis tetraptera. Unlike piperazine salts which only anesthetize the parasite, trichlorfon kills the worms, thus it is more efficacious. These data suggest that trichlorfon will not interfere with immunologic assays in mice and may be used safely in mice on experiment.

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